**The Dissertation Committee for Deena Marie Walker Certifies that this is the approved version of the following dissertation:**

# Molecular Mechanisms of Endocrine Disruption in the Hypothalamus Throughout the Life Cycle

**Committee:**

Andrea Gore, Supervisor

David Crews

Nigel Atkinson

Hans Hofmann

Marilyn McGinnis

# Molecular Mechanisms of Endocrine Disruption in the Hypothalamus Throughout the Life Cycle

**by**

**Deena Marie Walker, B.S.Bio**

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# Dedication

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# Molecular Mechanisms of Endocrine Disruption in the Hypothalamus Throughout the Life Cycle

Deena Marie Walker, Ph.D.

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Supervisor: Andrea C. Gore

Endocrine disrupting chemicals (EDCs) are compounds in the environment that interfere with hormone systems in the body. I investigated if gestational exposure to a known class of EDCs, polychlorinated biphenyls (PCBs), resulted in life long alterations in neuroendocrine function. My overall hypothesis was that prenatal PCB exposure would cause molecular and cellular changes to the developing hypothalamus that would manifest across development through differences in hypothalamic gene expression, molecular epigenetic modifications, and corresponding effects on sexual development. To perform this work, I characterized changes in gene expression in two regions of the hypothalamus required for reproductive function throughout the life cycle and measured changes in somatic markers associated with reproductive physiology and development. This approach allowed me to relate specific neuroendocrine changes back to altered reproductive function. First, I present normative data showing gene and hormone changes throughout development in male and female rats to use as a basis of comparison for my further studies on EDCs. Second, I investigated how gestational exposure to PCBs on

embryonic day 16 and 18 affected development of the hypothalamus through adulthood and caused corresponding changes in physiological functions. PCBs altered estrous cyclicity in females and delayed the timing of puberty in males. Developmental changes in gene expression were associated with sex, age and region of the hypothalamus. As a whole, the data suggested that gestational exposure to PCBs altered a network of hypothalamic genes and was associated with altered reproductive physiology. Finally, I extended my study farther along the life cycle to investigate if gestational exposure to PCBs altered the timing of reproductive aging in male and female rats. Few effects in males were observed. However, females exposed to PCBs had lower serum concentrations of LH on proestrus, and altered expression of numerous genes in the hypothalamus. These changes in gene expression were specific to the females' cycle status and the results provided novel insight into the molecular mechanisms underlying reproductive aging. Taken together, my dissertation resulted in a comprehensive profile of both normal hypothalamic developmental changes, as well as providing insight into endocrine disruption of hypothalamic gene networks from birth through aging.

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# GENERAL INTRODUCTION

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## Introduction to neuroendocrine systems

Vertebrates have two systems responsible for communication in the body: the nervous system and the endocrine system. The brain is not only the major regulatory element for both, but also a mediator between the two systems. The brain coordinates inputs from the environment with hormonal outputs from the endocrine system via the autonomic nervous system. Endocrine glands in the body are innervated by the autonomic nervous system, which controls glandular function in two ways: 1) by regulating blood flow into the gland or 2) by regulating the release of hormones from the gland. The hypothalamus, located at the base of the brain, is especially important for regulation of endocrine function as it serves as an interface between the nervous system and endocrine systems, and these endocrine functions that receive a driving input from the brain are called "neuroendocrine". There are two types of neuroendocrine outputs that are involved in the control of homeostatic processes. One of these outputs involves the release of peptide hormones, specifically vasopressin or oxytocin, from neurons that originate in the hypothalamus and terminate at a bloodstream located in the posterior pituitary gland. The second neuroendocrine output involves the release of peptide hormones collectively called hypothalamic releasing or inhibiting hormones into a blood system at the base of the

hypothalamus. This vasculature, called the portal capillary system, transports the hypothalamic peptides to the anterior pituitary gland, where they bind to specific pituitary receptors, a process that in turn results in the synthesis and release of secondary hormones. The anterior pituitary hormones then act at peripheral endocrine target glands to cause the release of tertiary hormones that affect various functions throughout the body. Along with these actions, tertiary endocrine hormones can exert negative feedback onto hypothalamic neurons or their inputs to suppress or modify the release of the initial hypothalamic hormones. Although all neuroendocrine systems exhibit negative feedback regulation, the reproductive hypothalamic-pituitary-gonadal (HPG) system of females has the unique ability to exert a positive feedback signal just prior to the time of ovulation (Gore and JL, 2003).

Endocrine hormones are essential for the normal development and function of the central nervous system (CNS). The prenatal and early postnatal period is particularly important in this regard as exposure to hormones during early development results in the normal organization and facilitation of many behaviors in the adult organism. Each axis of the neuroendocrine system is sensitive to organization at this time as evidenced by the fact that disruption of glucocorticoids (Mirescu and Gould, 2004; Mirescu et al., 2004), thyroid hormone (Ambrogini et al., 2005; Koibuchi et al., 1999) (Reviewed in (Schantz et al., 2001)) and sex steroid hormones (Reviewed (Wilson and Davies, 2007)) in the perinatal period result in long – term effects on brain function and behavior in the adult. The role of the brain as the primary regulator of neuroendocrine functions, together with the brain's

responsiveness to hormonal feedback, makes it an important organ to consider when studying endocrine disrupting chemicals (EDCs).

Endocrine-disrupting chemicals (EDCs) are "exogenous chemicals, or mixture of chemicals, that interferes with any aspect of hormone action" (Zoeller et al., 2012). Disruptions of hormone levels, their receptors or their actions in the brain through exposure to EDCs are not only disruptive to the structure and function of the brain itself but also to the regulation of hormones downstream from the brain. The mechanisms of endocrine disruption are diverse and complex; in the nervous system they probably involve actions through hormone receptors, the enzymes involved in the biosynthesis and degradation of hormones, and other aspects of hormone regulation. In addition, EDCs can be overtly neurotoxic through actions independent (or in addition to) hormone receptors, such as through actions neurotransmitter receptors, transporters and biosynthesis.

The discovery that the synthetic estrogen diethylstilbesterol (DES) can cause gynecological cancers and reproductive tract abnormalities in the daughters of mothers who took the drug during pregnancy was a landmark finding that demonstrated endocrine disruption in humans. It is now recognized that along with effects on the reproductive system, EDCs can disrupt numerous hormonal systems in the body including stress, thyroid, growth, and other metabolic hormones. This work will focus primarily on estrogenic EDCs and their effects on the mammalian brain in vivo. For information regarding other hormonal systems refer to the following reviews on thyroid (Zoeller, 2005); glucocorticoids (Moritz et al., 2005; Schantz et al., 2001); and androgens (Kelce and Wilson, 1997). As discussed in greater detail below, the mediation of estrogenic

effects of EDCs occurs through the two nuclear estrogen receptors, ERα and ERβ, both of which are abundant in brain, but which may be differentially affected by EDCs. There are also non-nuclear estrogen receptors that may be affected by EDCs. While there are numerous families of extensively studied estrogenic endocrine disruptors including, but not limited to phytoestrogens, organochlorine pesticides and plasticizers such as bisphenyl A, this work will focus on a class of prototypical endocrine disrupting chemicals called polychlorinated biphenyls (PCBs).

## Overview of Polychlorinated biphenyls (PCBs)

PCBs are synthetic compounds that were manufactured in the United States from the 1930's through 1970's. They are categorized into two groups, coplanar and non-coplanar, as per their molecular structure, and they exist in various states of chlorination. These structural features affect the ability of PCBs to bind to various hormone and neurotransmitter receptors and to act as agonists, antagonists, or mixed agonists/antagonists. Although banned in the 1970's, PCBs are environmentally persistent and widely dispersed throughout the globe, including in regions where PCBs were never used (e.g., the Arctic) due to populations of animals exposed to PCBs in industrial regions that migrate to non-industrial regions and enter the food chain. Additionally, PCBs are promiscuous compounds in that they exert actions on multiple classes of neurotransmitter and hormonal targets that are not always predictable. Some non-coplanar PCBs affect neurotransmitter systems (dopamine, serotonin, acetylcholine). In this way they may indirectly affect neuroendocrine functions. Other PCBs (usually coplanar) interact directly with endocrine systems to exert effects on thyroid hormone, glucocorticoids (Reviewed in

(Meserve et al., 1992; Schantz et al., 2001)) and sex steroid hormone levels. To make matters even more complicated, PCBs are both estrogenic and antiestrogenic as well antiandrogenic, an effect that is usually based on the structure of the specific congener (Reviewed in (Schantz and Widholm, 2001)). Finally, some "dioxin-like" PCBs may interact with orphan receptors such as the aryl hydrocarbon receptor (AhR; Reviewed in (Denison and Nagy, 2003)); this makes an understanding of PCB actions even more difficult since the endogenous ligand of the AhR is not currently known. It is this diversity of interactions that make the PCB compounds such an interesting subject for endocrine studies and a potentially dangerous compound for human exposure.

## PCBs actions in the brain during fetal and early postnatal development

The development of the brain begins in embryonic life and continues through puberty into adulthood. Neural development is a carefully regulated sequence of events that is controlled by several factors including genes, neurotransmitters, growth factors, hormones, and their interactions. Disruption of any one of these events by EDCs can have permanent effects on brain morphology and function, resulting in changes to physiology and behavior. To follow is a brief summary of the importance of normal pre- and early postnatal exposures to steroid hormones, to be followed by how early exposures to EDCs can disrupt these processes.

### BRAIN DEVELOPMENT, SEXUAL DIFFERENTIATION, AND THE ROLE OF STEROID HORMONES

The brain is sexually dimorphic, meaning that there are distinct differences in structure and morphology of regions between males and females. These differences are

permanent and determined during a critical period of sexual differentiation, largely through exposures to sex steroid hormones in fetal development or shortly after birth. In male mammals, much of sexual differentiation of the brain occurs via activation of the fetal testis to release the testicular hormone testosterone. Testosterone and its metabolite, estradiol, are responsible for sexual differentiation of the male brain through the actions of the metabolic enzyme aromatase, which converts testosterone to estradiol. The brain is an organ that has particularly high levels of aromatase, and together with the brain's high expression of both androgen and estrogen receptors, both endogenous and exogenous hormones may significantly impact neural function. Under normal developmental circumstances, early exposure to gonadal steroids lays the groundwork for the male physiology and behavior in the organism; the absence of such exposure results in a female phenotype (Reviewed in (Simerly, 1998; Wilson and Davies, 2007)).

The mechanisms for these effects of steroids in the nervous system are beginning to be understood. Steroid hormones, acting as transcription factors, determine which genes will be expressed in certain areas of the brain. Additionally, hormones may affect sexual differentiation at the cellular level by determining the brain's capacity to express sex steroid hormone receptors in a region and sex specific manner (Simerly, 1998) and cause other neurobiological changes such as dendrite outgrowth and synaptogenesis (Reviewed in (Matsumoto, 1991; Palanza et al., 1999)). In addition, structural effects of steroids, via apoptosis, have been observed in some areas of the brain. This is known to be a factor in the development of several sexually dimorphic nuclei in which hormones play an important role in both cell survival and programmed cell death (apoptosis). The balance of

these processes determines whether a brain region is masculinized, de-masculinized, feminized, or de-feminized, depending upon the sex and the type of exposure. For example, testosterone decreases cell death in three sexually dimorphic regions: the spinal nucleus of the bulbocavernosus (SNB) in male rats involved in penile erection (Johansen et al., 2004); the sexually dimorphic nucleus of the preoptic area (SDN-POA), thought to be involved in masculine sexual behavior in rats (Davis et al., 1996a); and the bed nucleus of the stria terminalis (BNST), involved in reproductive physiology and behavior and in affective behavior pathways (Reviewed in (Simerly, 2002)). On the other hand, testosterone increases cell death in the anteroventral periventricular nucleus (AVPV) (Reviewed in (Forger, 2006)) thought to regulate preovulatory GnRH release (Wiegand and Terasawa, 1982). Several of these aforementioned processes are not mediated by testosterone acting on its androgen receptor, but rather, by the metabolism of testosterone to estradiol and subsequent actions on estrogen receptors. Several relevant points need to be addressed in this regard. First, these early "organizational" effects of sex steroid hormones are necessary for the appropriate expression of sex-typical dimorphic behaviors later in life including reproductive behaviors such as the lordosis reflex in female rodents and mounting behavior in males. These latter behaviors require appropriate exposure to steroid hormones not only prenatally in the organizational period, but also pubertally, and the manifestation of these behaviors after puberty is referred to as the "activational" effects of steroid hormones. Second, early sex hormone exposure organizes other dimorphic non-reproductive behaviors such as infant play, aggression, learning, exploration and activity level (Reviewed in (Palanza et al., 1999)).

The fact that sex steroid hormones play a large role in normal sexual differentiation of the brain make early exposure to estrogenic/antiestrogenic and androgenic EDCs a potent dysregulator of brain function from genes to behavior. Notably, hormonal levels involved in controlling such events are extremely low (1 part per trillion) and the fetus is exceptionally sensitive to even the slightest shift in the hormonal milieu. Therefore, even the slightest imbalance can have exponential effects (Colbert et al., 1997). Because the events of early development are relatively well understood, and the developing fetus/neonate is especially sensitive to alterations in hormonal concentrations it is not surprising that many of the studies focusing on effects of EDCs on the brain examine their role in early development. To follow is such a discussion of the effects of PCB exposure in this period, a field often referred to as the "fetal (or developmental) basis of adult disease" (Reviewed in (Barker, 2003)).

### PCB EXPOSURE DURING EARLY BRAIN DEVELOPMENT

The estrogenic nature of PCBs has been known for decades. Exposure to these compounds during critical periods in development is especially detrimental to wildlife (Ferguson et al., 2000). Additionally, PCBs are lipophilic and so have the potential to bioaccumulate in adipose tissue. This is of particular concern when PCBs accumulate in tissues of pre-reproductive or reproductive females, because these compounds can be transferred to the developing offspring, either via the placenta or postnatally via lactation. Therefore, it is not surprising that many of the laboratory experiments examining the role of PCBs as endocrine disruptors have focused on developmental exposure and their long-

term effects on estrogen responsive genes, cellular processes, structural alterations and behaviors.

**Gene Expression, Protein Expression and Brain Morphology**

Numerous studies have shown that PCBs can have long-term effects on gene expression throughout the body after developmental exposure (Desaulniers et al., 2005; Desaulniers et al., 2009; Shimada et al., 2010). Studies from our laboratory showed that young adult rats exposed to Aroclor 1221 (A1221) (a mixture of lightly chlorinated PCB congeners) during development displayed altered sexually dimorphic expression of key genes and proteins involved in reproductive function in the AVPV, a sexually brain dimorphic region involved in the control of female ovulation (Dickerson et al., 2011b; Salama et al., 2003). Other studies focusing on aromatase activity have found that gestational exposure to a mixture of PCBs decrease aromatase activity in the hypothalamus of male pups (Hany et al., 1999). Additionally, PCBs may act on neurotransmitter systems in the brain, as gestational exposure to A1254 has been shown to alter dopamine (Seegal et al., 2005), acetylcholine (Corey et al., 1996; Juarez de Ku et al., 1994) and serotonin (Morse et al., 1996) systems throughout the adult brain.

The consequences of EDCs altering protein expression and enzymatic activity during development include potentially profound neurodevelopmental effects throughout the brain. The effects of PCBs exposure on structural (non-sexually dimorphic) changes have been observed throughout the brain and are associated with locomotor (Nguon et al., 2005; Roegge et al., 2004) and cognitive (Seegal et al., 1994) deficits in adults exposed to PCBs in utero.

**Reproductive Physiology and Behavior**

Several studies from our laboratory and others have shown that gestational exposure to PCBs alters reproductive physiology. Lyche et al (2004), reported that PCB153, but not PCB126, decreased prepubertal LH concentrations, delayed puberty and resulted in higher progesterone concentrations during ovulation (luteal phase) in 9 month old female goats exposed during gestation and lactation (Lyche et al., 2004). Additionally, Hany et al. (Hany et al., 1999) found that treatment with a reconstituted mixture of PCBs throughout gestation resulted in a decrease in serum testosterone levels in adult male rats. Studies from our laboratory have found that serum progesterone and testosterone were decreased in adult males exposed to A1221 (Dickerson et al., 2011b) and serum LH was increased on estrus in adult females (Steinberg et al., 2008). A1221 has also been shown to alter the timing of puberty in males and females (Dickerson et al., 2011b; Gellert, 1978b), eye opening, estrous cyclicity (Dickerson et al., 2011b) and may advance reproductive senescence in females (Gellert, 1978b; Gellert and Wilson, 1979). While further investigation is necessary to determine the mechanisms these effects, it is clear that gestational exposure to PCBs results in long-term alterations in reproductive physiology.

These physiological effects may manifest in altered reproductive behavior and reduced fertility. For example, neonatal exposure to A1254 reduces sexual receptivity (lordosis quotient) while A1221 has no effect (Chung et al., 2001). However, when treatment includes pre and postnatal exposure, A1254 decreases sexual motivation and A1221 decreases sexual receptivity (Chung and Clemens, 1999; Steinberg et al., 2007).

These data suggest that timing of exposure is an extremely important parameter to consider when analyzing the effects of EDCs on sexual behavior.

PCBs also alter sexually dimorphic behaviors. For example, when rats are exposure to a reconstituted mixture that is similar to that found in human breast milk an increase in sweet preference in males was observed. This sexually dimorphic behavior is usually increased in females, suggesting that gestational and lactational exposure can feminize sexually dimorphic behaviors in adult rats (Hany et al., 1999; Kaya et al., 2002).

**CONCLUSIONS**

Taken together, these data suggest that perinatal exposure to PCBs have consequences throughout the brain and these effects are, in many cases, persistent. However, it is difficult to interpret these results further as the studies discussed differ in many experimental parameters (i.e., dose, age and duration of exposure, nature of compound, etc.). The few conclusions that can be made suggest that PCBs have effects on neurotransmitter release and metabolism, reproductive function and alter normal development (both somatic and cognitive). Further studies are needed to conclusively determine how early exposure to PCBs affect brain development and function.

To address this I designed 3 experiments to determine if gestational exposure to PCBs results in sex and age specific effects on the reproductive neuroendocrine axis. Chapter 1 provides a description of gene expression profiles in the hypothalamus throughout postnatal development (neonatal period – adulthood) in untreated male and female rats as well as characterizes changes in sex steroid hormones in the same animals. This was necessary to provide us with a comprehensive developmental profile of 48

neuroendocrine genes and serum hormone changes, something that was lacking in the current literature. This also enabled me to investigate sex and age specific relationships in these endpoints and identify novel interactions not previously reported (Walker et al., 2012). Chapter 2 investigated if gestational exposure to PCBs disrupts development of two hypothalamic nuclei necessary for reproductive function in males and females as well as identifies functional outcomes of exposure to EDCs. I addressed this by characterizing gene expression changes in the hypothalamus in siblings exposed to PCBs *in utero* and relating those to the alterations in reproductive function and physiology, such as the altered timing of puberty and estrous cyclicity in males and females respectively. Chapter 3 expands this study into the paradigm of reproductive aging by relating gene expression changes observed in the hypothalamus to the peripheral manifestations of reproductive senescence. Taken together, these experiments provide a comprehensive profile of neuroendocrine gene expression throughout the life cycle as well as identifying those brain regions, genes and hormonal changes that are associated with reproductive transitions and may be sensitive to disruption by gestational exposure to EDCs. Finally, I was able to identify novel targets of EDCs and determine important players in reproductive function that had not been previously reported.

# CHAPTER 1

## Profiles of Neuroendocrine Gene Expression Throughout Postnatal Development in Female and Male Rats

Significant portions of this chapter were published previously in: Walker DM, Kirson D, Perez LF and Gore AC (2012) Molecular Profiling of Postnatal Development of the Preoptic Area and Mediobasal Hypothalamus in Female and Male Rats, Biology of Reproduction, Oct 3 (Epub ahead of print).

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**Abstract**

Reproductive function is highly dynamic during postnatal developmental. Here, we performed molecular profiling of gene expression patterns in the hypothalamus of developing male and female rats to identify which genes are sexually dimorphic, to gain insight into a more complex network of hypothalamic genes, and to ascertain dynamic changes in their relationships with one another and with sex steroid hormones during development. Using a low-density PCR platform, we quantified mRNA levels in preoptic area (POA) and medial basal hypothalamus (MBH), and assayed circulating estradiol, testosterone, progesterone at six ages from birth through adulthood. Numerous genes underwent developmental change, particularly postnatal increases, decreases, or peaks/plateaus at puberty. Surprisingly, there were few sex differences; only *Esr1*, *Kiss1* and *Tac2* were dimorphic (higher in females). Cluster analysis of gene expression revealed sexually dimorphic correlations in the POA but not the MBH from P30 to P60. Hormone

measurements showed few sex differences in developmental profiles of estradiol; higher levels in progesterone only after P30; and a developmental pattern of testosterone with a nadir at P30 followed by a dramatic increase through P60 (males only). Furthermore, bionetwork analysis revealed that hypothalamic gene expression profiles and their relationships to hormones undergo dynamic developmental changes that differ considerably from adults. These data underscore the importance of developmental stage in considering effects of hormones on the regulation of neuroendocrine genes in the hypothalamus. Moreover, the finding that few neuroendocrine genes are sexually dimorphic highlights the need to consider postnatal development from a network approach that allows assessment of interactions and patterns of expression.

## INTRODUCTION

For an individual to be able to reproduce, its reproductive system must be in synchrony with favorable external stimuli and internal factors that permit and even optimize reproductive success. For example, serum hormone levels and patterns of release, adequate nutritional status, and physiological and psychological maturity affect reproductive capacity, as do light cycles, seasonal variations, and immune function (Ebling, 2005). Of all of the body's systems, the reproductive system is one of the most highly dynamic in the context of the life cycle, with reproductive competence not being attained until after a complex and often protracted postnatal developmental and pubertal process has occurred. Much of this coordination is orchestrated through a hypothalamic neural and glial network that converges upon the gonadotropin-releasing hormone (GnRH) neurons that provide the final output from the brain to the pituitary gland. For

example, the onset of puberty is initiated by an increase in the pulsatile release of GnRH from these neurons, but GnRH neurons are already mature at birth. This has led to the hypothesis that neural and glial inputs to GnRH neurons are responsible for initially clamping GnRH release at low levels prepubertally, then enabling pulsatile GnRH release to increase postnatally through the progression of puberty (Ojeda et al., 2010c; Terasawa and Fernandez, 2001).

The hypothalamus is structurally and functionally sexually dimorphic (Gorski et al., 1980), something that plays out as sex differences in GnRH release, hypothalamic neural functioning, and reproductive behavior. Sexual differentiation of the hypothalamus is strongly influenced by differential exposure of male and female brains to sex steroid hormones in early life (Wilson and Davies, 2007). However, GnRH neurons themselves do not express most sex steroid hormone receptors (Hrabovszky et al., 2001; Wintermantel et al., 2006). Therefore, effects of developmental steroids on this neural network are mediated by afferent inputs to GnRH neurons. Excitatory inputs thought to regulate GnRH include kisspeptin (Kiss1), neurokinin B (Tac2) and glutamate, whereas inhibitory inputs include the opioid, dynorphin (Colledge et al., 2010; Ojeda et al., 2010b). GABA exerts both excitatory and inhibitory influences and this balance may contribute to developmental regulation of GnRH (Herbison and Moenter, 2011). Transcription factors that activate or repress gene expression also play a key role (Ojeda et al., 2010b). Nevertheless, it remains unclear how these and other factors interact to result in the attainment of reproductive competence.

There is considerable literature on how peripheral sex hormones, especially estradiol and testosterone, regulate gene expression in the hypothalamus. Most of this work was conducted in adults, using models of castration and/or hormone treatments (Guerra-Araiza et al., 2002; Handa et al., 1996; Lauber et al., 1991a; Lustig et al., 1989; McAbee and DonCarlos, 1999b; Navarro et al., 2004; Navarro et al., 2009; Navarro et al., 2011b; Simerly and Young, 1991; Smith et al., 2006; Tessier et al., 2000; Yuri and Kawata, 1991). A separate body of work has addressed how circulating serum hormones are involved during sexual differentiation of the brain during early life (Wilson and Davies, 2007). However, there is surprisingly little research on the relationship between hormones and gene expression throughout postnatal development in an intact model (Walker et al., 2009). Considering that the brain remains sensitive to steroid hormones postnatally (Sisk and Foster, 2004), during which time there are large changes in both hormone and neurotransmitter release, this is an important gap in research.

While previous studies have measured gene expression in the hypothalamus, and characterized circulating hormones, much of this work has been cross-sectional, used few ages, did not compare the sexes, or studied one or a few genes. The literature for individual genes and hormones varies widely due to these experimental differences. Thus, our goal was to provide a more unified perspective on hormones, gene expression, and developmental sex differences through quantification of 48 hypothalamic genes and assaying serum concentrations of sex steroid hormones in male and female animals across postnatal development.

## MATERIALS AND METHODS

### Animals

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin Institutional Animal Use and Care Committee. Sex differences in neuroendocrine gene expression and serum hormone concentrations were investigated throughout postnatal development on postnatal days (P) 1, 5, 15, 30, 45, and 60. Sprague-Dawley rats were purchased from Harlan Laboratories (Houston, TX) and impregnated in house. Dams were allowed to give birth and on the day after birth (P1) litter composition and birth weights were recorded. The study was designed to distribute male and female littermates across as many different ages as possible. For earlier time points (P1, 5), two same-sex siblings were pooled to ensure enough RNA and serum for all assays. For later time points (P15, 30, 45, 60) individual rats were used. At P1 and P5, samples from 2 littermates were pooled to get a targeted N=8 (see figures for final numbers). A total of 10 litters were used to achieve this number. Pups were weaned on P21 and housed in same-sex groups (2-3 per cage) with food and water provided ad libitum, at a constant temperature (21- 22 C) with a partially reversed light cycle (lights on at 2300h). A secondary sex characteristic of the onset of puberty was monitored daily (preputial separation in males and vaginal opening in females). After vaginal opening, females were subjected to daily vaginal smears.

### Tissue Collection and Storage

Rats were euthanized by rapid decapitation between 10:30 and 11:30 h. Postpubertal females were euthanized on the day of proestrus as indicated by vaginal cell

cytology. Brains were removed and the preoptic area (POA) and medial basal hypothalamus (MBH) were dissected using standard brain landmarks as described (Dickerson et al., 2008; Jakubowski et al., 1991) and snap frozen on dry ice. Trunk bloods were collected, allowed to clot, and serum was separated via centrifugation (1500 X g for 5 min). Tissues and serum were stored at -80C until use.

**RNA Extraction**

RNA was extracted from frozen POA and MBH tissues using an in-house double detergent lysis buffer system (Jakubowski et al., 1991). Samples were homogenized using a 22-gauge needle and 1cc syringe, cytoplasmic RNA was treated with proteinase K, extracted with phenol chloroform, and precipitated in ethanol. Resuspended RNA was treated with 1 U of TURBO® DNase (Applied Biosystems Inc., Foster City, CA) to rid samples of genomic DNA. All samples were run on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) to assess RNA purity, integrity and concentration.

**Taqman® Microfluidic Real-time PCR Cards**

Samples were run on custom-designed microfluidic 48-gene PCR cards (Applied Biosystems Inc, Foster City, CA), with specific gene assays chosen based on a priori hypotheses and published reports on their importance in neuroendocrine function, timing of puberty, steroid hormone responsiveness, and sex differences (46 genes of interest and 2 housekeeping genes; a complete list is provided in Supplemental Table 3.1). Because it is a Taqman PCR-based card, it does not require further PCR validation (which would be redundant). Nevertheless, we have previously validated this assay by comparing it with conventional gene-by-gene PCR assays and had excellent replication of results from

identical samples run by the two methods (Walker et al., 2009). Inter- and intra-sample variability on the cards is low (Walker et al., 2009) and we have since been using single samples because of the high cost of the assay together with its very low variability in our hands. Cytoplasmic RNA (2$\mu$g) was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA) according to manufacturer's protocol. Product was stored at -20 until use at which time samples were diluted 1:10 before PCR reactions were conducted.

Real-time RT-PCR was carried out on an ABI 7900 using Taqman® Universal Mastermix (Applied Biosystems, Foster City CA) and the following run parameters: 50C for 2 minutes, 95C for 10 minutes, 45 cycles of 94.5C for 30 seconds and 59.7C for 1 minute. Relative expression was determined for each sample using the comparative Ct Method (Livak and Schmittgen, 2001; Pfaffl, 2001; Schmittgen and Livak, 2008). Samples were normalized to Gapdh as described previously (Gore et al., 2011; Walker et al., 2009) and calibrated to the median delta-Ct of the group with the lowest expression to determine fold-change in expression for each gene.

**Serum hormone assays**

Serum hormone concentrations were measured using radioimmunoassay (RIA) for estradiol ($E_2$), progesterone ($P_4$) and testosterone (T) (Beckman Coulter, Webster, TX) according to the manufacturer's recommended protocols. Samples were run in duplicate if possible. For the youngest animals (P1 and 5) serum was pooled when necessary to ensure there was sufficient serum for all assays. Samples having a CV of 10% or greater were either rerun when possible or dropped from analysis ($E_2$ = 5 samples dropped, T = 1

sample dropped, $P_4$ = 2 samples dropped). In a few cases, there was not enough serum to run all serum hormones for the P1 animals so serum from other P1 animals was used for the testosterone and estradiol assays to maintain sample size as close to n=8 as possible.

For the estradiol assay, two assays were run with assay sensitivity of 2.2 pg/ml and intra-assay variability of 4.22% (14 samples) and 4.06% (90 samples). Inter-assay variability was 11.49%. For the testosterone assay, two assays were run with assay sensitivity of 0.08 ng/ml and intra-assay variability of 2.47% (88 sample) and 3.2% (27 samples). Inter-assay variability was 36.86%. While the inter-assay variability is high, each assay had representatives from each age and sex and there was not a significant difference between the two runs in serum concentrations obtained, and group data show small standard error bars suggesting that the variability did not affect the validity of serum results. These testosterone levels were also in line with those reported previously in our laboratory (Walker et al., 2009) and others (Dohler and Wuttke, 1975). For the progesterone assay, one assay was run with assay sensitivity of 0.12 ng/ml and intra-assay variability of 2.96%.

**Statistics**

For gene expression data, statistics were performed using relative expression for each sample. Multiple regression analysis was conducted using PASW® software (IBM, Armonk NY) to compare each endpoint (genes and hormones) using age and sex as independent variables. For those endpoints where a significant main effect of age or a sex by age interaction was observed a Tukey-Kramer post hoc analysis was performed to determine specific differences between each group. If data did not meet the assumptions

for multiple regression, data were transformed (natural log or square root) and reanalyzed. In a few cases, transformed data did not meet assumptions for statistical analysis by multiple regression. In those cases, data were analyzed using a Kruskal-Wallis test followed by Mann-Whitney test between each group. For hormone concentrations an effect was considered significant at $p<0.05$. For gene expression data a Benjamini and Hochberg False-Discovery Rate correction (Benjamini and Hochberg, 1995; Benjamini and Hochberg, 2000) was used to correct our p-values in order to account for the large number of variables measured. Gene expression data were tested for outliers using the z-score of the residuals from the initial regression. A data point was considered an outlier if the residual was greater than 2.5 standard deviations from initial line of best fit. For hormone concentrations, data were tested for outliers using the Grubbs' outlier test. Confirmed outliers were excluded from final analysis.

To examine possible relationships between gene expression and serum hormones throughout development, data were analyzed using a bootstrap technique (Efron and Tibshirani, 1993). Briefly, original paired data was resampled with replacement for 1000 repetitions, and Pearson's correlation coefficient was computed on each of these new bootstrapped data sets to build a distribution of coefficients. Significance of Pearson's correlation coefficient for each gene/gene or gene/hormone interaction was determined from the bootstrapped distributions. Only those correlation coefficients that survived a Benjamini and Hochberg False Discovery Rate (Benjamini and Hochberg, 1995; Benjamini and Hochberg, 2000) at a p-value $< 0.05$ were considered significant. The

bootstrap and subsequent analyses were performed using Matlab (The Mathworks, Natick MA) software.

Hierarchical cluster analysis and heatmaps were performed using Multiple Experiment Viewer V4.8.1 (TM4.org) and clusters were validated using R statistical packages.

## RESULTS

### Sex Differences in Developmental Profiles of Neuroendocrine Genes in the POA and MBH

Expression of 48 genes was measured by real-time RT-PCR throughout development in the POA and MBH of females and males using the Taqman low-density array. Of these, only 3 genes exhibited any sexual dimorphism, with a significant age by sex effect for *Esr1* and *Kiss1* in the POA (Figure 1.1A, C), and main effects of sex for *Esr1*, *Kiss1*, and *Tac2* in the MBH.

In the POA, *Esr1* expression was significantly greater (~3-fold) in females than males early in development (P1 – P15; $p<0.05$), a difference that disappeared after adolescence (P30 – P60; Figure 1.1A). Additionally, a significant main effect of age was found for *Esr1* expression, with developmental decreases in both female and male POA, albeit a much smaller magnitude of change in males. *Kiss1* mRNA (Figure 1.1C) expression was also sexually dimorphic in the POA, with significantly greater expression (~3-fold) in females when compared to males from P30 – P45 ($p<0.01$). Additionally, age related increases were observed for *Kiss1* in both males and females, (~30-fold increase in females from P5 – 30, and ~10-fold increase in males from P5 – P15). *Tac2* mRNA

expression in the POA (Figure 1.1E) demonstrated a significant age-related increase, but no sex difference. Post-hoc analysis revealed a significant increase (~5-fold) in expression from P1 – P15 followed by another significant 2-fold increase from P30 – P45 ($p<0.01$) in both sexes.

In the MBH, there were significant main effects of sex ($p<0.001$) and age ($p<0.001$) for *Esr1*, *Kiss1* and *Tac2* (Figure 1.1B, D, F) but no significant interactions. *Esr1* expression was greater in developing females than males, with significant increases from P1 – P15 (~2-fold). *Kiss1* (Figure 1.1D) expression increased significantly from P1 – P15 (~2-fold) and P5 – P30 (~3-fold) in males and females. Females had significantly greater *Kiss1* expression than males, especially early in postnatal life. There was a trend for an interaction of sex and age ($p=0.02$); however, this effect did not survive a false-discovery rate correction. Finally, *Tac2* (Figure 1.1F) expression was greater in females than males ($p<0.001$) and expression increased ~3-fold from P5 – P15 in both sexes.

**Developmental Changes in Neuroendocrine Gene Expression in the POA and MBH**

The real-time PCR array contains 2 internal controls for normalization: Gapdh, and 18s. Both controls displayed small (~20%) but significant age related changes. Therefore, we took a conservative approach: only genes that survived a false discovery rate correction and displayed greater than a 2-fold change in expression were included for further analysis. (This was also taken into consideration in the gene analysis described above for *Esr1*, *Kiss1* and *Tac2*.) In the POA and MBH, 31 and 29 genes, respectively, met these criteria. In order to maintain consistency with past publications that used the same technology, all gene expression was normalized to Gapdh expression (Walker et al.,

2009). For graphic purposes, genes were categorized into related functional groups (Figure 1.2). Because no sex differences were observed, data were collapsed across the sexes for graphing and analysis. Of those genes undergoing substantial developmental change, the most common patterns were a developmental decrease or developmental increase through puberty, with regional differences for a subset of genes.

In order to determine the relationships among specific genes across postnatal development, hierarchical cluster analysis was conducted using MeV 4.8.1 and validated using R statistical packages. Clustergrams were constructed using MeV 4.8.1 software (Figure 1.3) for each brain region and sex using the correlation coefficients to express similarity and the average linkage method. Validated clusters are indicated in red. In general, the genes clustered into two broad groups identified from inspection of developmental profiles (Figures 1.1 and 1.2): those that decreased from P1 and those that increased from P1. Clusters by sex and age reveal sex differences in the POA (Figure 1.3A) from P30 to P60 that were not observed in the MBH (Figure 1.3B). In the POA (Figure 1.3A), males and females clustered together from P1 to P15, but from P30 to P60 the sexes segregated into two separate clusters. In the MBH (Figure 1.3B), females and males clustered together by age from P1 through P60 indicating that while there were few sex differences in the expression patterns of gene expression, there were sex differences in the correlation of genes in the POA but not the MBH.

**Sex Differences in the Developmental Profiles of Serum Hormone Concentrations**

Serum concentrations of estradiol (Figure 1.4A), testosterone (Figure 1.4B) and progesterone (Figure 1.4C) were measured in the same developing rats as those used for

gene expression. In both sexes, estradiol concentrations were significantly greater on P15 when compared to any other time point ($p<0.05$) but there were no sex differences. In males, testosterone exhibited a nadir at P30, followed by a dramatic increase to P60. Mann-Whitney post-hoc analysis revealed that concentrations were significantly greater in males when compared to females on all days but P30. In females, serum testosterone was significantly greater on P15 when compared to all other days. Finally, serum testosterone was significantly greater on P60 in males than all other ages, regardless of sex ($p< 0.05$). For progesterone, significant age by sex interactions as well as significant main effects of age and sex ($p<0.001$), were detected. Progesterone concentrations were identical in males and females from P1 to P30. From P45 to P60, progesterone levels were significantly greater in females than males, as well as all other ages in both males and females ($p < 0.01$).

**Integration of genes and hormones as developmental networks**

To examine the relationship between gonadal hormones and hypothalamic gene expression throughout development we used the network analysis platform Cytoscape (Cline et al., 2007; Kohl et al., 2011; Yeung et al., 2008) to generate networks based on significant Pearson correlation coefficients between hormones and relative gene expression in each brain region. Data were first collapsed across the sexes for each age in each region to identify relationships that change throughout postnatal development regardless of sex (Figures 1.5 and 1.6). In order to display the networks in a legible format the networks for P1 and P5 in both the POA and MBH are not shown in Figures 1.5 and

1.6. For information regarding changes on P1 and P5 in the POA and MBH please see (Walker et al., 2012).

In the POA, the highest number of significant correlations was detected on P1 (data not shown) and P30 (Figure 1.5B). On P15, there were a number of negative correlations, many of them with sex steroid hormones and their receptors, an effect not observed on other ages. From P30 to P60 in the POA, the majority of the correlations were positive. Finally on P60, *Gnrh1* was negatively correlated with 7 genes; four are growth factors or their receptors, two are associated with neurotransmitters (*Gabbr2* and *Grin2c*), and one is the steroidogenic enzyme, aromatase p450 (*Cyp19a1*). In the MBH, there were more correlations on P5 (data not shown) than on any other day. Additionally on P15, *Pdyn* was negatively correlated with a number of genes, and on P45 *Hsd17b2* was negatively correlated with 7 genes, six of which were neurotransmitters.

## FIGURES AND TABLES

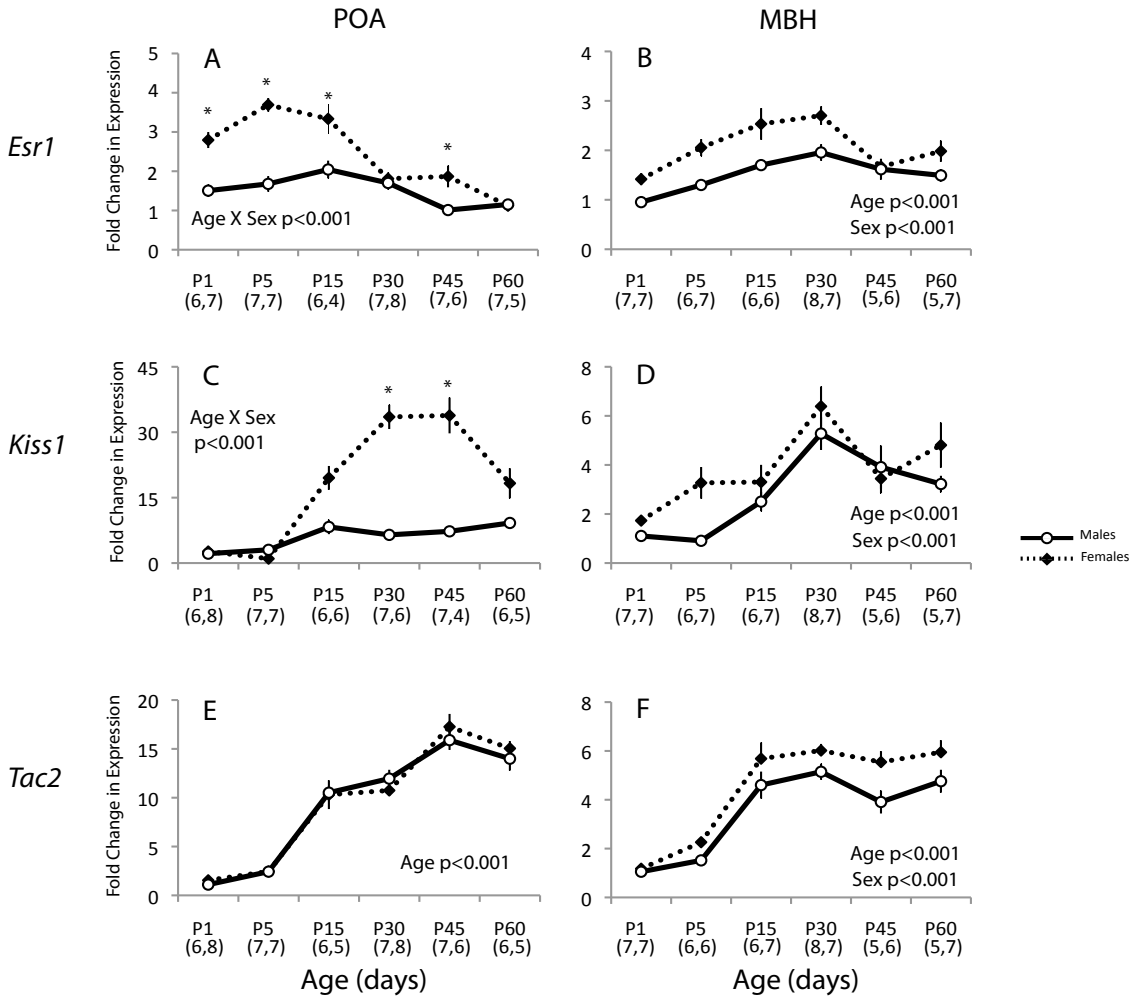
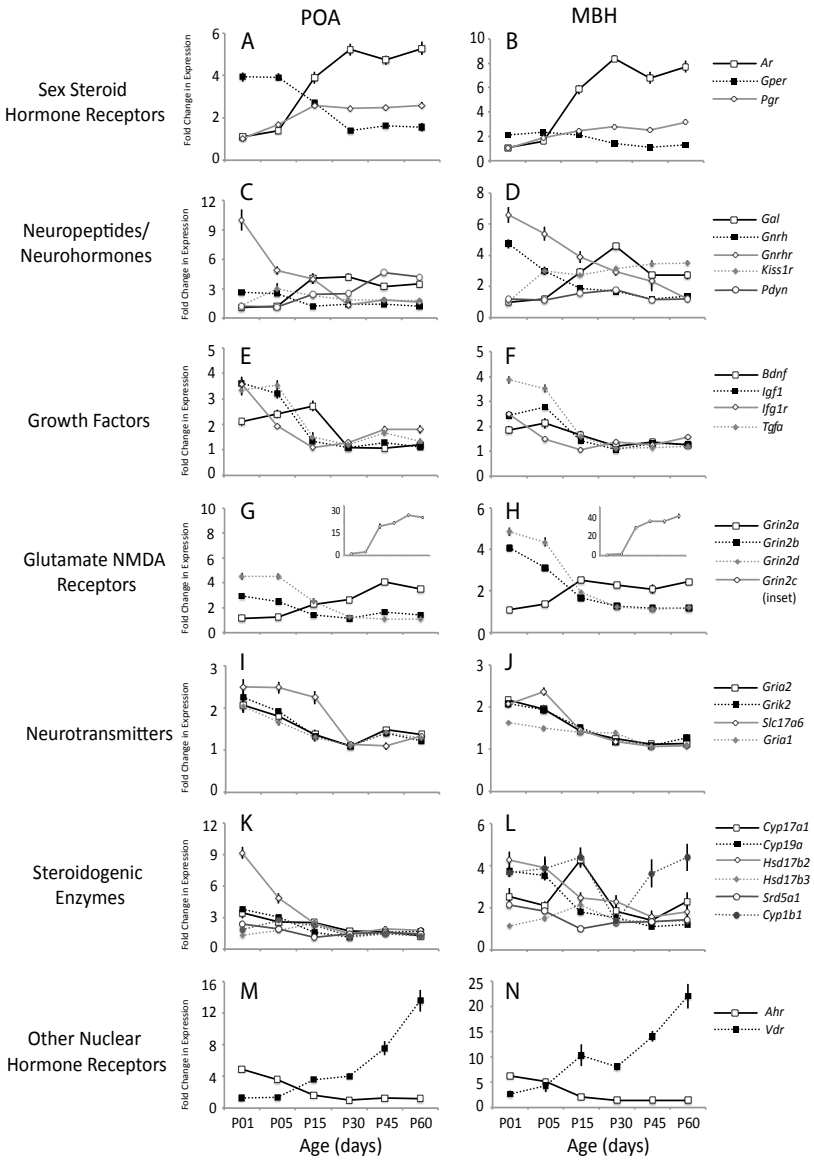



Figure 1.1: Expression of three sexually dimorphic genes is shown in the POA & MBH

Data are presented for postnatal days (P) 1, 5, 15, 30, 45, & 60) in males (solid lines) and females (dotted lines). Data are expressed as fold-change in relative expression, with the age/sex having the lowest expression level set at 1.0. *Esr1* and *Kiss1* had a significant interaction of age and sex in the POA ($p<0.001$), and there were significant main effects of age ($p<0.001$) and sex ($p<0.001$) in MBH. *Tac2* was sexually dimorphic in the MBH only ($p<0.001$), and underwent a significant developmental increase in both regions ($p<0.001$). Significant sex differences for each age are indicated by *. Numbers of rats here and in other figures are shown parenthetically for males (left) and females (right).

Figure 1.2: Developmental profiles of non-sexually dimorphic genes in the POA & MBH.




Data are shown combined for males and females, as there were no sex differences, and results are displayed as relative fold-change. Genes were graphed as functional groups. In panels G and H, because *Grin2c* underwent substantially higher-fold change than other NMDA receptors, its presentation is broken out into the inset.

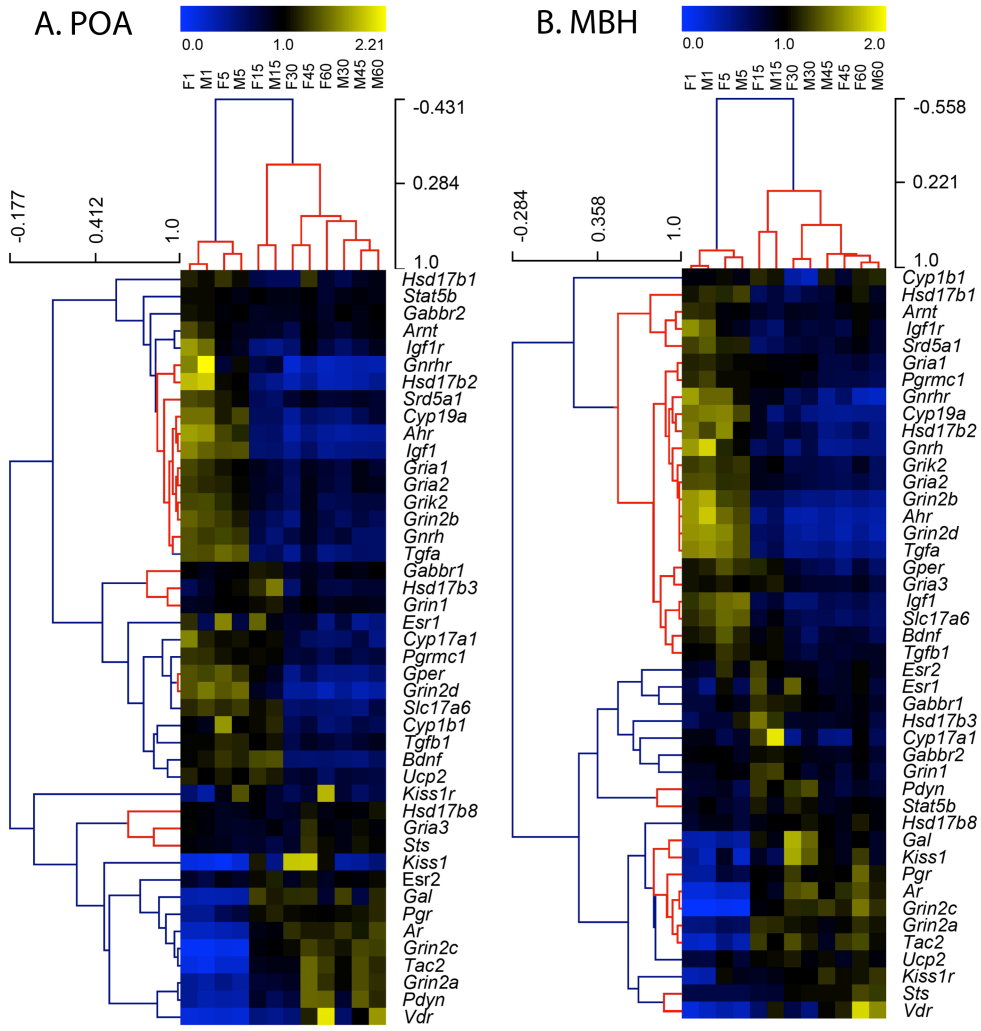



Figure 1.3: Hierarchical cluster analysis of gene expression in the POA and MBH

Analysis was conducted using the correlation coefficients to express similarity and the average linkage method was conducted using the Multiple Experiment Viewer V4.8.1 (TM4.org), and clusters were validated using R statistical packages to reveal genes that grouped by expression patterns in the POA (A) and MBH (B). Data are displayed as highest expression in yellow and lowest expression in blue and validated clusters are indicated in red brackets for genes (to the left of the clustergrams) and for sex and age (above the clustergrams). For both POA and MBH, genes clustered into two general expression patterns; those increasing from P1 (bottom) or decreasing from P1 (top). In the POA, males and females clustered together by age from P1 to P15. However, from P30 to P60 the females and males clustered within the sexes. In the MBH, males and females clustered by age throughout development.

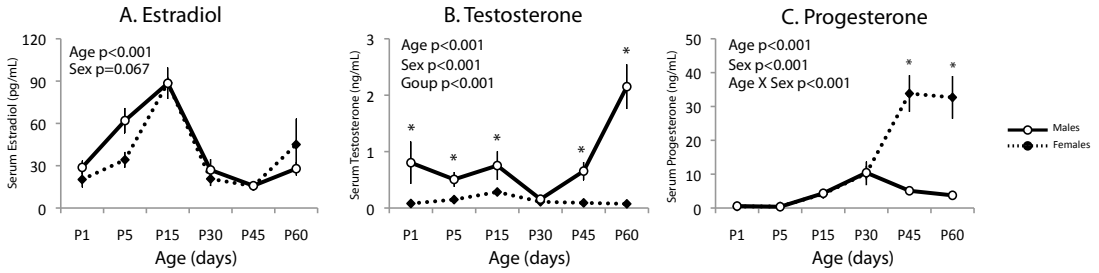



Figure 1.4: Serum hormone changes throughout development in male and female rats.

Serum estradiol (A), testosterone (B) and progesterone (C) concentrations are shown in male (solid line) and female (dotted line) rats throughout postnatal development. Serum estradiol (A) changed significantly in males and females throughout postnatal development ($p<0.001$) and was highest on P15 in both sexes. Serum testosterone (B) changed significantly with age ($p<0.001$). In males, serum T dropped to a nadir on P30 and then increased into adulthood whereas in females, serum T peaked at P15 ($p<0.05$). Serum progesterone had significant changes by age ($p<0.001$); sex ($p<0.001$) and a significant interaction of sex and age ($p<0.001$). In both sexes, serum $P_4$ increased until P30 but diverged thereafter, with levels falling in males and increasing in females. * Denotes significant sex differences at each age.

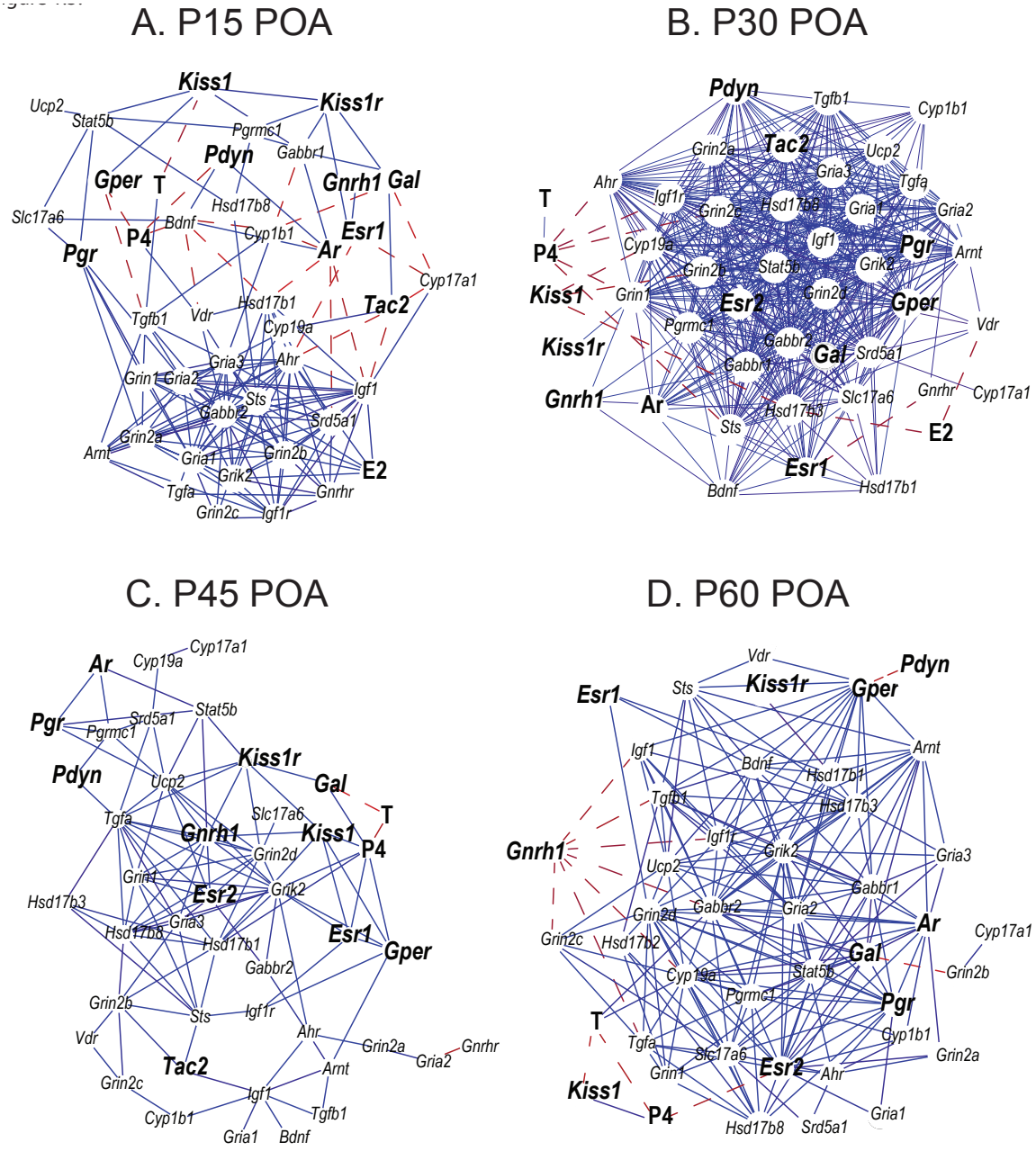



Figure 1.5: Networks of gene and hormone correlations in the POA.

Data are shown for those developmental days with the greatest change (P15, 30, 45 and 60). Positive correlations are indicated as blue solid lines and negative correlations are indicated as red dotted lines. Thirteen specific genes and hormones are indicated by the larger font and bolded text as a selected subset (see text for description).

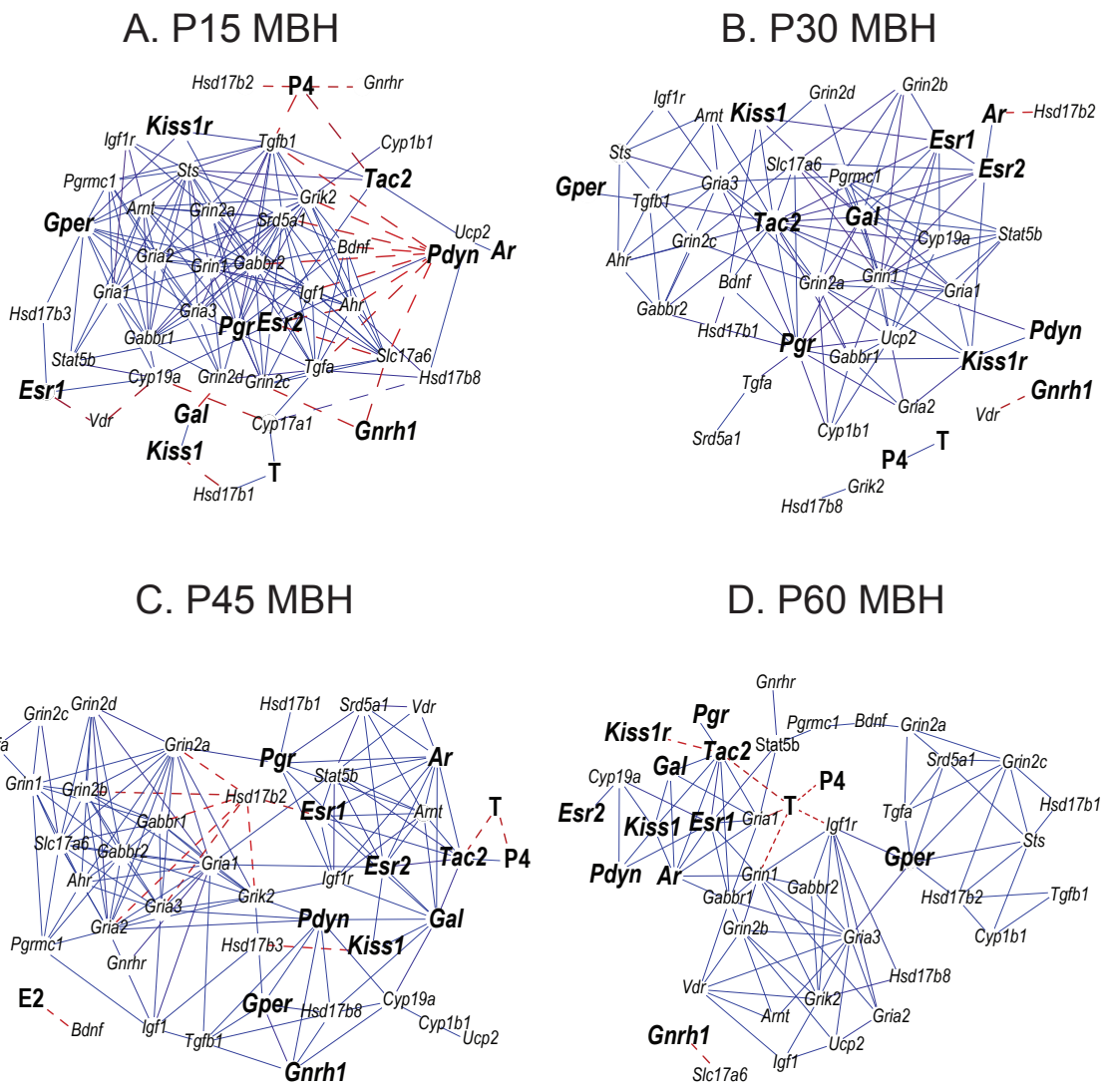



Figure 1.6: Networks of gene and hormone correlations in the MBH.

Data are shown for those developmental days with the greatest change (P15, 30, 45 and 60). Positive correlations are indicated as blue solid lines and negative correlations are indicated as red dotted lines. Thirteen specific genes and hormones are indicated by the larger font and bolded text as a selected subset (see text for description).

| POA | | | | | |
|---|---|---|---|---|---|
| **Gene Name** | **Gene Symbol** | **Average** | **Age** | **Sex** | **Age X Sex** |
| cytochrome P450, family 17, subfamily A, polypeptide 1 | *Cyp17a1* | 4.02 | **< 0.001** | 0.476 | 0.654 |
| hydroxysteroid (17-beta) dehydrogenase 2 | *Hsd17b2* | 8.37 | **< 0.001** | 0.951 | 0.913 |
| gonadotropin-releasing hormone receptor | *Gnrhr* | 38.10 | **< 0.001** | 0.037 | 0.808 |
| vitamin D receptor | *Vdr* | 62.14 | **< 0.001** | 0.281 | 0.815 |
| hydroxysteroid (17-beta) dehydrogenase 1 | *Hsd17b1* | 136.92 | **< 0.001** | 0.061 | 0.126 |
| hydroxysteroid (17-beta) dehydrogenase 3 | *Hsd17b3* | 157.05 | **< 0.001** | 0.019 | 0.274 |
| KiSS-1 metastasis-suppressor | *Kiss1* | 266.38 | **< 0.001** | **< 0.001** | **< 0.001** |
| cytochrome P450, family 1, subfamily B, polypeptide 1 | *Cyp1b1* | 328.01 | **0.003** | 0.351 | 0.654 |
| estrogen receptor 2 | *Esr2* | 620.04 | **0.006** | 0.879 | 0.328 |
| aryl hydrocarbon receptor | *Ahr* | 638.38 | **< 0.001** | 0.571 | 0.658 |
| KiSS-1 receptor | *Kiss1r* | 1228.81 | **< 0.001** | 0.975 | N/A |
| cytochrome P450, family 19, subfamily A, polypeptide 1 | *Cyp19a* | 1304.59 | **< 0.001** | 0.01 | 0.627 |
| brain-derived neurotrophic factor | *Bdnf* | 1317.43 | **< 0.001** | 0.849 | 0.95 |
| estrogen receptor 1 | *Esr1* | 1614.99 | **< 0.001** | **< 0.001** | **< 0.001** |
| androgen receptor | *Ar* | 1923.49 | **< 0.001** | 0.407 | 0.948 |
| G protein-coupled estrogen receptor 1 | *Gper* | 2367.39 | **< 0.001** | 0.221 | 0.294 |
| progesterone receptor | *Pgr* | 3084.07 | **< 0.001** | 0.377 | 0.215 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2C | *Grin2c* | 3710.58 | **< 0.001** | 0.718 | 0.595 |
| steroid sulfatase | *Sts* | 3896.34 | **< 0.001** | 0.126 | 0.495 |
| gonadotropin-releasing hormone 1 | *Gnrh1* | 4132.63 | **< 0.001** | 0.018 | 0.442 |
| steroid-5-alpha-reductase | *Srd5a1* | 4864.10 | **< 0.001** | 0.683 | 0.447 |
| hydroxysteroid (17-beta) dehydrogenase 8 | *Hsd17b8* | 5036.75 | 0.468 | 0.944 | 0.264 |
| aryl hydrocarbon receptor nuclear translocator | *Arnt* | 5052.37 | **< 0.001** | 0.404 | 0.3 |
| transforming growth factor, beta 1 | *Tgfb1* | 5129.44 | **< 0.001** | 0.604 | 0.752 |
| insulin-like growth factor 1 | *Igf1* | 5342.64 | **< 0.001** | 0.889 | 0.838 |
| galanin prepropeptide | *Gal* | 6425.92 | **< 0.001** | 0.896 | 0.416 |
| transforming growth factor, alpha | *Tgfa* | 7565.17 | **< 0.001** | 0.628 | 0.155 |
| tachykinin 2/neuronkinin b | *Tac2* | 8689.02 | **< 0.001** | 0.31 | 0.35 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2A | *Grin2a* | 8887.78 | **< 0.001** | 0.625 | 0.192 |
| uncoupling protein 2 | *Ucp2* | 11400.67 | **< 0.001** | 0.436 | 0.102 |
| glutamate receptor, ionotropic, AMPA 3 | *Gria3* | 13549.35 | **< 0.001** | 0.075 | 0.331 |
| insulin-like growth factor 1 receptor | *Igf1r* | 14080.70 | **< 0.001** | 0.221 | 0.494 |
| glutamate receptor, ionotropic, kainate 2 | *Grik2* | 15752.70 | **< 0.001** | 0.415 | 0.491 |
| prodynorphin | *Pdyn* | 22599.81 | **< 0.001** | 0.767 | 0.752 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2D | *Grin2d* | 22673.04 | **< 0.001** | 0.654 | 0.123 |
| gamma-aminobutyric acid (GABA) B receptor, 1 | *Gabbr1* | 23348.65 | **< 0.001** | 0.131 | 0.664 |
| solute carrier family 17 member 6 | *Slc17a6* | 27005.37 | **< 0.001** | 0.464 | 0.45 |
| signal transducer and activator of transcription 5B | *Sta5b* | 32038.25 | **0.008** | 0.776 | 0.618 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2B | *Grin2b* | 33098.07 | **< 0.001** | 0.171 | 0.183 |
| gamma-aminobutyric acid (GABA) B receptor, 2 | *Gabbr2* | 44716.49 | **0.012** | 0.278 | 0.265 |
| glutamate receptor, ionotropic, AMPA 1 | *Gria1* | 48646.20 | **< 0.001** | 0.692 | 0.577 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 1 | *Grin1* | 79806.02 | **0.008** | 0.956 | 0.872 |
| glutamate receptor, ionotropic, AMPA 2 | *Gria2* | 99445.15 | **< 0.001** | 0.38 | 0.901 |
| progesterone receptor membrane component 1 | *Pgrmc1* | 217005.91 | **< 0.001** | 0.034 | 0.481 |

Table 1.1 Abundance of the genes on the Taqman real-time PCR array in the POA.

To compare abundance of each gene, the average relative expression for each gene was determined by using the lowest expressed genes on the plate as a calibrator and calculating relative expression for all genes for each animal in the study. Differences in abundance of the genes in the POA are sorted from lowest (green) to highest (red) in abundance. The original p-value is listed and those genes that survived a False-discovery rate correction are indicated in bold. For those genes for which non-parametric analysis was conducted, a p-value could not be obtained for sex by age interactions (indicated by N/A).

| MBH | | | | | |
|---|---|---|---|---|---|
| **Gene Name** | **Gene Symbol** | **Average** | **Age** | **Sex** | **Age X Sex** |
| cytochrome P450, family 17, subfamily A, polypeptide 1 | *Cyp17a1* | 2.57 | **<0.001** | 0.171 | 0.334 |
| hydroxysteroid (17-beta) dehydrogenase 2 | *Hsd17b2* | 5.41 | **<0.001** | 0.145 | 0.918 |
| gonadotropin-releasing hormone receptor | *Gnrhr* | 23.05 | **<0.001** | 0.553 | 0.548 |
| vitamin D receptor | *Vdr* | 41.72 | **<0.001** | 0.2 | 0.151 |
| hydroxysteroid (17-beta) dehydrogenase 1 | *Hsd17b1* | 83.09 | **<0.001** | 0.978 | 0.224 |
| cytochrome P450, family 1, subfamily B, polypeptide 1 | *Cyp1b1* | 90.85 | **0.001** | 0.806 | N/A |
| hydroxysteroid (17-beta) dehydrogenase 3 | *Hsd17b3* | 167.81 | **<0.001** | 0.474 | N/A |
| gonadotropin-releasing hormone 1 | *Gnrh1* | 208.05 | **<0.001** | 0.989 | 0.428 |
| estrogen receptor 2 | *Esr2* | 274.03 | **0.004** | 0.021 | 0.122 |
| aryl hydrocarbon receptor | *Ahr* | 376.03 | **<0.001** | 0.996 | N/A |
| cytochrome P450, family 19, subfamily A, polypeptide 1 | *Cyp19a* | 378.29 | **<0.001** | 0.265 | N/A |
| KiSS-1 metastasis-suppressor | *Kiss1* | 549.55 | **<0.001** | **<0.001** | 0.02 |
| KiSS-1 receptor | *Kiss1r* | 580.63 | **<0.001** | 0.616 | 0.664 |
| estrogen receptor 1 | *Esr1* | 705.93 | **<0.001** | **<0.001** | 0.334 |
| androgen receptor | *Ar* | 1626.38 | **<0.001** | 0.067 | 0.689 |
| G protein-coupled estrogen receptor 1 | *Gper* | 1632.56 | **<0.001** | 0.273 | 0.177 |
| brain-derived neurotrophic factor | *Bdnf* | 1702.74 | **0.001** | 0.346 | 0.141 |
| progesterone receptor | *Pgr* | 2061.15 | **<0.001** | 0.39 | 0.223 |
| steroid sulfatase | *Sts* | 2294.53 | **<0.001** | 0.437 | 0.963 |
| aryl hydrocarbon receptor nuclear translocator | *Arnt* | 2851.35 | **<0.001** | 0.151 | 0.891 |
| insulin-like growth factor 1 | *Igf1* | 2918.35 | **<0.001** | 0.143 | 0.438 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2C | *Grin2c* | 3031.44 | **<0.001** | 0.381 | N/A |
| transforming growth factor, beta 1 | *Tgfb1* | 3136.11 | **<0.001** | 0.497 | 0.382 |
| steroid-5-alpha-reductase | *Srd5a1* | 3413.19 | **<0.001** | 0.706 | 0.448 |
| tachykinin 2/neuronkinin b | *Tac2* | 3642.77 | **<0.001** | **<0.001** | 0.257 |
| hydroxysteroid (17-beta) dehydrogenase 8 | *Hsd17b8* | 3964.84 | **0.003** | 0.73 | N/A |
| transforming growth factor, alpha | *Tgfa* | 4133.47 | **<0.001** | 0.167 | 0.503 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2A | *Grin2a* | 4257.73 | **<0.001** | 0.45 | N/A |
| glutamate receptor, ionotropic, AMPA 3 | *Gria3* | 5575.57 | **<0.001** | 0.094 | 0.339 |
| insulin-like growth factor 1 receptor | *Igf1r* | 6829.86 | **<0.001** | 0.055 | 0.634 |
| glutamate receptor, ionotropic, kainate 2 | *Grik2* | 9545.89 | **<0.001** | 0.885 | 0.215 |
| uncoupling protein 2 | *Ucp2* | 10079.10 | **<0.001** | 0.434 | 0.093 |
| galanin prepropeptide | *Gal* | 11745.90 | **<0.001** | 0.019 | 0.459 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2D | *Grin2d* | 12219.98 | **<0.001** | 0.822 | N/A |
| gamma-aminobutyric acid (GABA) B receptor, 1 | *Gabbr1* | 14683.43 | **<0.001** | 0.134 | 0.607 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 2B | *Grin2b* | 17799.08 | **<0.001** | 0.907 | 0.632 |
| prodynorphin | *Pdyn* | 18002.06 | **<0.001** | 0.778 | 0.721 |
| gamma-aminobutyric acid (GABA) B receptor, 2 | *Gabbr2* | 18292.57 | **0.012** | 0.343 | 0.226 |
| signal transducer and activator of transcription 5B | *Stat5b* | 20893.90 | **0.007** | 0.144 | 0.458 |
| glutamate receptor, ionotropic, AMPA 1 | *Gria1* | 26192.21 | **<0.001** | 0.213 | 0.668 |
| solute carrier family 17 member 6 | *Slc17a6* | 36264.58 | **<0.001** | 0.462 | N/A |
| glutamate receptor, ionotropic, AMPA 2 | *Gria2* | 43672.21 | **<0.001** | 0.307 | 0.84 |
| glutamate receptor, ionotropic, N-methyl D-aspartate 1 | *Grin1* | 51638.93 | **<0.001** | 0.487 | N/A |
| progesterone receptor membrane component 1 | *Pgrmc1* | 131080.27 | **<0.001** | 0.009 | 0.234 |

Table 1.2 Abundance of the genes on the Taqman real-time PCR array in the MBH.

To compare abundance of each gene, the average relative expression for each gene was determined by using the lowest expressed genes on the plate as a calibrator and calculating relative expression for all genes for each animal in the study. Differences in abundance of the genes in the MBH are sorted from lowest (green) to highest (red) in abundance. The original p-value is listed and those genes that survived a False-discovery rate correction are indicated in bold. For those genes for which non-parametric analysis was conducted, a p-value could not be obtained for sex by age interactions (indicated by N/A).

## DISCUSSION

In the current study, we profiled a network of neuroendocrine-related hypothalamic genes and serum hormones in the context of postnatal development of the two sexes. Our results revealed novel relationships and surprisingly few sex differences, leading us to believe that the developing hypothalamus has very different hormone-dependent and -independent properties from those in adults. A number of published studies have identified sex differences in gene expression (Cao and Patisaul, 2011; DonCarlos and Handa, 1994; Kurian et al., 2010b; McAbee and Doncarlos, 1999a, b; Poling et al., 2012; Takumi et al., 2011) protein expression (MacLusky et al., 1979; Yokosuka et al., 1997) and epigenetic changes (Kurian et al., 2010a; Kurian et al., 2010b; Schwarz et al., 2010) in the brain during postnatal development. However, our study differs from all other published work in several fundamental ways. Using a real-time PCR panel, we were able to study a network of 48 neuroendocrine genes implicated in the hypothalamic control of pubertal development, allowing us to do specific hypothesis testing about important genes implicated in brain sexual differentiation (e.g., *Esr1*, *Kiss1*, *Tac2*) and at the same time, identify potentially novel pathways not systematically studied in the hypothalamus. Second, we utilized developing male and female littermates distributed across 6 ages chosen to span perinatal, prepubertal, peripubertal and postpubertal periods. This type of systematic approach is important because it eliminates confounds of over-reliance on littermates within a single age group. Third, we compared rostral (POA) and caudal (MBH) hypothalamic regions to enhance understanding of tissue specificity in expression. Fourth, we measured the three major gonadal steroids in these

same animals, something rarely done in a single study containing both sexes (Dohler and Wuttke, 1974, 1975) and not previously in the context of hypothalamic gene expression. Therefore, we believe that we are the first to determine not only how numerous neuroendocrine genes change over time but also whether and how their relationships change in a sex-specific manner, together with their associations with serum hormones.

### *Esr1*, *Kiss1* and *Tac2* mRNAs are sexually dimorphic in the hypothalamus

One of the most surprising results of this study was that only three of the 48 genes analyzed showed significant sexual dimorphisms: *Esr1*, *Kiss1* and *Tac2* – all necessary for reproductive function and the pubertal transition (Colledge et al., 2010; Ojeda et al., 2010a; Ojeda et al., 2010c). Furthermore, these genes are co-expressed in the same kisspeptinergic neurons in parts of the hypothalamus. More specifically, in the POA, nearly all of *Kiss1* neurons co-express *Esr1*, and in the MBH there are a population of *Kiss1*-expressing neurons that also express *Tac2* and *Pdyn*, often referred to as KNDy neurons. The KNDy neurons also express *Esr1* and are sensitive to fluctuations in estradiol (Reviewed in (Garcia-Galiano et al., 2012)). Our findings of robust developmental changes in a sex- and region-specific manner support the importance of these genes in the maturation of the hypothalamus.

In both hypothalamic regions studied, *Kiss1* and *Tac2* increased developmentally. By contrast, *Esr1* decreased in the POA, and in the MBH it increased to P30, then decreased from P30 to P60. While the decrease in *Esr1* expression was surprising, a recent study observed a similar expression pattern (Cao and Patisaul, 2011) in several hypothalamic nuclei [anteroventral periventricular nucleus (AVPV), medial POA, arcuate

nucleus (ARC), and ventromedial nucleus]. Interestingly, knocking out *Esr1* specifically in kisspeptin neurons advanced the timing of puberty in females (Mayer et al., 2010) suggesting that there may be a functional significance to the developmental decrease seen in the current study. The sexual dimorphism of *Kiss1* and *Esr1* found herein replicated previous studies in young animals (Cao and Patisaul, 2011; DonCarlos and Handa, 1994; Poling and Kauffman, 2012; Takumi et al., 2011). However, one novel finding of our study is that *Kiss1* and *Esr1* are not sexually dimorphic in the adult (P60) POA. Numerous studies have shown that *Kiss1* is greater in the female AVPV (Reviewed in (Kauffman, 2009)) of adult animals. However, in the present study *Kiss1* expression is only significantly greater in the female POA on P30 and 45 (with a trend on P15; p=0.073). Additionally, it should be noted that we do not observe sex differences in expression of numerous genes that been reported to be sexually dimorphic in the rostral hypothalamus (Reviewed in (Simerly, 2002)) including, but not limited to, *Esr1* (Orikasa and Sakuma, 2003) and androgen receptor (McAbee and DonCarlos, 1998, 1999b). We hypothesize that these differences are likely the result of differences in experimental design including the time of day the animals were euthanized (our animals are euthanized prior to lights out), cycle stage of the females (euthanized here on proestrus) and gonadal status (our rats were intact). Furthermore, the POA and MBH dissections utilized here include several smaller subnuclei that may be differentially regulated by age, sex, and/or hormones something that we are currently addressing in ongoing work using hypothalamic microdissections. Even within more discrete hypothalamic regions, it is also necessary to recognize the fundamental heterogeneity of nervous system tissue – with a mixture of neuronal

phenotypes, glial cells, blood vessels, etc. – requiring interpretation of all data in light of this mix of cells. Therefore, a limitation of gene expression work on homogenized tissue is the ability to determine specific cellular changes, something that needs to be accomplished by other methodologies such as immunohistochemistry, *in situ* hybridization, or single cell profiling. Despite these potential limitations, our results in POA and MBH highlight the complex and differential interplay between sex steroid hormones, neuroendocrine genes, and hypothalamic region in response to circadian cycles and gonadal status. Indeed, recent studies in females suggest that *Kiss1* expression is under circadian control in the AVPV of female rodents (males were not investigated) (Robertson et al., 2009; Smarr et al., 2012). Future studies should include intact animals euthanized at different circadian intervals to gain an understanding of these complex interactions.

There is little published research on dimorphisms of *Tac2*, found here in the MBH but not the POA of our developing rats. While previous work reported that *Tac2* is not sexually dimorphic in the KNDy neurons in the ARC (Kauffman et al., 2009; Navarro et al., 2011a; Navarro et al., 2009), there are the same caveats as mentioned above for *Kiss1*. Furthermore, *Tac2* in the MBH and potentially in the KNDy neurons themselves may also be sensitive to circadian/estrous regulation. In support of this hypothesis, *Tac2* expression in the ARC is greatest on proestrus in females (Rance and Bruce, 1994) (again, males were not investigated). While the functional role of the KNDy neurons in the ARC is only beginning to be understood, Navarro et al. have hypothesized that *Tac2* may function as a pacemaking signal for *Kiss1* release from these neurons (Navarro et al., 2011a; Navarro et al., 2009), a hypothesis that is supported by our current study.

**Developmental non-sexually dimorphic changes in gene expression**

The PCR platform identified an additional 28 or 26 genes that changed significantly and displayed a greater than 2-fold change throughout postnatal development in either the POA or MBH, respectively (Figure 1.2). While there were some differences in the extent of fold changes observed between the POA and MBH, many of the genes shared similar expression patterns throughout postnatal development. However, there were notable differences between the two regions. In the POA but not MBH, two genes changed significantly with developmental age: *Pdyn* increased 4-fold (Figure 1.2C & D) and *Gria1* (Figure 1.2I & J) decreased 2-fold from P1 to P60. Additionally, two steroidogenic enzymes, *Cyp1b* and *Cyp17a1* had different expression patterns in the MBH and POA. *Cyp1b1* metabolizes 17β-estradiol and *Cyp17a1* catalyzes the conversion of cholesterol to progestins (Dumas and Diorio, 2011). Differences in these enzymes might indicate a drop in local available steroid neurohormones. There is an intriguing body of work suggesting that polymorphisms in Cyp17 alleles may be associated with the advancement of puberty in young women (Lai et al., 2001; Mitchell et al., 2008), implicating this gene in the tempo of reproductive maturation.

Interestingly, the few genes on our array implicated in the inhibition of GnRH secretion, namely, *Pdyn* (MBH only), *Gabbr1*, *Gabbr2*, *Tgfb1* and *Pgrmc1* showed little change in expression throughout development (data not shown with exception of *Pdyn*). However, genes associated with excitatory inputs to the GnRH system changed substantially in the current study. Previously, it has been hypothesized that the onset of puberty is regulated by an increase in excitatory and a decrease in inhibitory inputs onto

the GnRH system (Ojeda et al., 2010c; Terasawa and Fernandez, 2001). The current findings provide evidence that the mechanisms regulating these inputs may differ in the hypothalamus during development. Importantly, in our study the developmental expression patterns seen for stimulatory inputs undergo dynamic change, suggesting that there is some "reorganization" of the excitatory inputs and not just a simple increase in stimulation to the GnRH neurons. We hypothesize that during postnatal development, stimulatory signals may switch to regulation by neuropeptides that are more responsive to peripheral changes in sex steroid hormones, including *Kiss1*, *Gal* and *Tac2*, all of which increase dramatically during this period. While this hypothesis needs further evaluation, our data provide intriguing preliminary evidence of this possibility. Our data also do not negate the potential importance of inhibitory neurotransmitters and their relationship to postnatal development, as post-transcriptional changes may occur to these factors that are not necessarily paralleled by mRNA changes.

**Cluster analysis reveals sex differences in expression patterns**

We used cluster analysis to evaluate sex and age differences in correlations of genes as a way to predict common regulators of expression (Figure 1.3). In general, genes in the POA and MBH clustered according to two expression profiles: those that increased from P1 (bottom cluster) or decreased from P1 (top cluster). Interestingly, in the POA males and females cluster together by age on P1, P5 and P15 but the two sexes cluster separately from P30 to P60 suggesting that sex differences in correlations in the POA emerge after P30. This effect was not observed in the MBH where the males and females of each age cluster together throughout development. Taken together these data provide

evidence that sexual dimorphisms in expression in the POA are age dependent and that the developmental stage of the animals should be taken into account in conducting sex differences studies.

**Sex differences in serum hormone concentrations**

Developmental concentrations of sex hormones have previously been described in rodents (Dohler and Wuttke, 1974, 1975; Lieberburg et al., 1979; Piacsek and Goodspeed, 1978; Poling and Kauffman, 2012; Saksena and Lau, 1979; Vomachka et al., 1981; Walker et al., 2009), but to our knowledge only one previous publication has characterized sex differences in the 3 major gonadal hormones across development within a single study (Dohler and Wuttke, 1975). Concentrations of $E_2$, $P_4$ and T in our rats were quite similar to those from the literature, and with only one exception (discussed below), postnatal patterns and sex differences were also consistent with the published literature.

Other reports of postnatal developmental changes in circulating $E_2$ demonstrate that levels are at their highest early in life and then experience a decrease, but the exact age at which the peak occurs varies among studies. Two groups (Dohler and Wuttke, 1975; Konkle and McCarthy, 2011) reported that $E_2$ levels were highest on P1, declined over the next few days, then had a smaller peak at about day 9, falling again thereafter. When we measured serum $E_2$ levels at P1, 5, 15, 30, 45 and 60 in males, our results matched those of these two other groups, with an overall decrease from P1 through P30 (Walker et al., 2009). By contrast, in the current study, $E_2$ concentrations were highest in both sexes on P15. The developmental changes in serum $E_2$ observed here were also similar to those reported for hamsters (Vomachka et al., 1981) and male rats (Saksena and Lau, 1979). The overall

conclusion that we draw from current and past work is that serum estradiol concentrations are similar in developing female and male rats, and that they undergo a decrease as the animals mature. While we do not know the explanation for differences among studies, they may be attributable to intrauterine position, prenatal litter size and sex ratio; postnatal litter composition, including pup numbers and sex ratio after culling of litters; time of day that animals were euthanized; or differences in hormone binding proteins such as alpha-fetoprotein, among others.

Serum testosterone (T) in the current study was greater in males than females for most of postnatal development with the exception of P30 when male T concentrations dropped to undetectable levels. This finding in males replicated our and others' previous reports (Dohler and Wuttke, 1975; Walker et al., 2009). Additionally, we noted a significant peak of T in females on P15, similar to earlier work (Dohler and Wuttke, 1975).

Likewise, our results on serum $P_4$ were consistent with the literature (Dohler and Wuttke, 1974, 1975). We observed significant sex differences in serum P4 after puberty, with much higher $P_4$ concentrations observed in females on P45 and P60. Again similar to previous findings, serum $P_4$ peaked in male rats on P30, the same day when serum T is undetectable in males (Dohler and Wuttke, 1974, 1975; Walker et al., 2009). Taken together, these data provide important developmental time points that may be important for sex differences in the timing of puberty.

**Network analysis of genes and hormones reveals novel interactions during postnatal development**

As in our previous study (Walker et al., 2009), we noticed that a number of genes reported to be regulated by steroid hormones displayed developmental patterns that diverged from predicted relationships to specific serum hormone concentrations. For example, *Pgr* (progesterone receptor) is regulated by *Esr1* and its ligand $E_2$ in numerous areas of the female brain but not the male brain (Guerra-Araiza et al., 2002; Lauber et al., 1991b, c). However, in our study serum $E_2$ decreased from P15 to P45, a time when *Pgr* is unchanged in both the male and female POA and MBH. Therefore, we used Cytoscape to assemble networks of genes and hormones with significant correlations according to developmental age in the hopes of generating new hypotheses about how genes and hormones interact in an intact rat model throughout development. Furthermore, because expression of a gene is not necessarily paralleled by expression of its protein product, results on genes that undergo developmental change and/or sex differences should be followed up by western blotting or immunohistochemistry. In fact, we hope that our gene bionetwork results will serve as a resource to guide future experimentation in this arena. Here, to simplify our model, we combined data from the two sexes and highlighted thirteen selected endpoints (indicated in larger font and bold text in Figures 1.5 and 1.6) to facilitate the identification of regulatory networks. However, we present the entire network so that readers can search for relationships among other endpoints of interest. This network analysis showed surprising interactions among hormones and sex steroid hormone receptors that challenge the traditional dogma of how sex steroid hormone receptors are regulated by their ligands (Walker et al., 2009). For example, at no point in

development is *Pgr* positively correlated with $E_2$ (in fact, on P5 in the POA, *Pgr* is negatively correlated with $E_2$). With the exception of the P15 POA, it is rare that any of the serum hormones serves as a regulatory hub in either the POA or the MBH. As a whole, the network analysis shows the complexity of sex steroid hormone interactions with gene expression in the brain of developing rats.

Because sex steroid hormone receptors are necessary for sexual differentiation of the brain (Reviewed in (Simerly, 2002)) and reproductive function (Reviewed in (Garcia-Galiano et al., 2012)) we expected that they would be major hubs in our networks throughout postnatal development. However, this was rarely the case. One exception was *Esr2* (estrogen receptor beta), which was a hub at every age in the POA except P15. Another consistent relationship that was observed throughout development in both the POA and MBH was between *Pgr* with *Ar* (androgen receptor), and $P_4$ with T. On P1, P45 and P60 in the POA and P1, P5 and P45 in the MBH, *Ar* and *Pgr* are positively correlated, but at P45 and P60, serum T and $P_4$ are negatively correlated. Together with similar results from a previous study (Walker et al., 2009), our data indicate that there is a strong relationship between *Ar* and *Pgr* that deserves further investigation.

## SUMMARY AND CONCLUSIONS

Previous work investigating relationships among hormones and gene expression has relied almost entirely upon models of gonadectomy, with or without hormone treatment, with most work done on adults. Our studies of intact developing male and female rats represent a different perspective and revealed several surprising results. First, we found that there are relatively few sex differences in the expression patterns of genes

throughout postnatal development. Second, the subset of genes that was sexually dimorphic (*Esr1*, *Kiss1*, *Tac2*) represents a defined category of molecules implicated in the control of postnatal reproductive development (Colledge et al., 2010). Third, we identified novel relationships of sex steroid hormones, their receptors, and neuropeptides in the POA and MBH indicating a complex mechanism of regulation that is dependent on the developmental time point and sex. The correlation and network analysis approaches can be used to generate hypotheses for future testing in studies of postnatal development of the hypothalamus.

# CHAPTER 2

## Neuroendocrine disruption of postnatal development caused by prenatal PCBs in female and male rats

**ABSTRACT**

Polychlorinated biphenyls (PCBs) are a class of synthetic compounds developed in the 1930s for industrial use. Although use of PCBs was banned in the US in the 1970s, many congeners remain in the environment and human exposure is ubiquitous through contact with contaminated soil, water and food sources. Studies from our laboratory and others have found that gestational exposure to Aroclor 1221 (A1221), a commercially available mixture of lightly chlorinated PCBs, has long term effects on the neuroendocrine reproductive system including altering serum hormone concentrations, estrous cyclicity, gene and protein expression, female reproductive behaviors, the timing of puberty and/or reproductive senescence and sexual differentiation of the hypothalamus. We tested the hypothesis that early life exposure A1221 alters development of the hypothalamus that results in altered reproductive function. Pregnant Sprague-Dawley rats were injected on embryonic day 16 and 18 with vehicle (DMSO), A1221 (1mg/kg) or estradiol benzoate (EB) (50$\mu$g/kb) as an estrogenic control. After birth, developmental milestones such as the timing of puberty and estrous cyclicity were recorded throughout the life cycle. On postnatal days (P) 15, 30, 45, and 90, 1 male and 1 female from each litter was euthanized. Punches from two brain regions important for reproductive function were collected: anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) and frozen for assessment of mRNA expression of a panel of 48 neuroendocrine genes and analysis of DNA methylation. We determined gestational exposure to A1221 delayed the timing of

puberty in males and disrupted estrous cyclicity in females. This was associated with disruption of gene expression in the AVPV of females and ARC of males. We also identified novel targets of PCBs, including two clock genes *Arntl* and *Per2*, which displayed a male-typical expression profile in the female AVPV. However, DNA methylation of regulatory regions of 2 genes (*Per2* and *Ar*) displaying sex specific disruptions of expression in the AVPV was not disrupted.

## INTRODUCTION

In order for an individual to attain reproductive competency a myriad of tightly coordinated processes must occur beginning in embryonic development and continue throughout adulthood. While the entire hypothalamic-pituitary-gonadal (HPG) axis undergoes dynamic changes, it is the hypothalamus that integrates internal and external stimuli, including metabolic status, serum hormone concentrations, stressors, social conditions, circadian indicators and immune function, which lead to and optimize reproductive capacity in adulthood (Ebling, 2005). This dynamic process is organized during the embryonic/postnatal period and activated during the pubertal process, which culminates in the increased synthesis and release of gonadotropin-releasing hormone (GnRH), the obligatory decapeptide for reproductive function (Reviewed in (Ojeda et al., 2010c)). However, GnRH neurons are mature at birth (Wray, 2002). Therefore, it is hypothesized that a complex neural and glial network that converges onto these neurons is responsible for 1) suppressing GnRH release prepubertally, 2) enabling the increase in pulsatile release during the pubertal process and 3) maintaining its release in adulthood (Terasawa and Fernandez, 2001). In an effort to understand how this neural network

coordinates the downstream effectors of reproductive function, postnatal developmental changes in gene expression (Cao and Patisaul, 2011; McAbee and DonCarlos, 1998; Navarro et al., 2004; Navarro et al., 2012; Sladek et al., 2004; Tena-Sempere et al., 2001; Walker et al., 2009; Walker et al., 2012), protein expression (Davis et al., 2004; Quadros et al., 2002; Sladek et al., 2004; Yokosuka et al., 1997), epigenetic processes (Nugent et al., 2011; Schwarz et al., 2010), and morphology (Abeliovich and Beal, 2006; Davis et al., 1996a; Davis et al., 1996b) have been characterized in the hypothalamus of the developing rodent.

The hypothalamic neuronal network controlling reproduction is also sexually dimorphic (Wilson and Davies, 2007). These differences result in sex differences in response to peripheral cues and culminate in male- and female-typical reproductive function, physiology and developmental processes (Clark et al., 1993; Pignatelli et al., 2006; Sisk and Zehr, 2005). Sexual differentiation of the brain is highly influenced by sex steroid hormones during a critical period of gestation/early postnatal development. Although many sexual dimorphisms do not manifest until much later in life following an activational period, it is during this organizational sensitive period that the brain is permanently masculinized/defeminized or feminized/demasculinized (Davis et al., 1996a; Davis et al., 1996b; Jacobson et al., 1980; Rhees et al., 1990a, b). Mounting evidence suggests that sexual differentiation of the brain involves epigenetic mechanisms, including DNA methylation and chromatin remodeling, which "program" gene expression in a male/female typical manner through the actions of sex steroid hormone with their receptors (Reviewed in (Matsuda KI et al., 2012 and McCarthy M 2009)). Therefore,

exposure to endocrine disrupting chemicals (EDCs) during this critical period of brain developmental can result in life-long effects on reproductive physiology and behavior by "programming" the expression of sexually dimorphic genes that are necessary for female and male typical reproductive function.

EDCs are defined as "an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action" (Zoeller et al., 2012) and include a variety of plastics, plasticizers (phthalates and BPA), pharmaceuticals (e.g., DES), pesticides (DDT, methoxychlor) and industrial contaminants (PCBs, dioxins), among others. Here, we investigated if exposure to an environmentally relevant dose of lightly chlorinated PCBs, Aroclor 1221 (A1221), alters the developmental profiles of gene expression in two nuclei in the hypothalamus known to regulate reproductive function – the anteroventral periventricular (AVPV) and arcuate (ARC) nucleus - and identify relationships between gene expression and peripheral changes in endocrine tissues and reproductive physiology. The AVPV and ARC are subregions of the gross hypothalamic dissections investigated in Chapter 1. Here we used micropunches of hypothalamic regions in order to characterize expression changes in more refined regions of the rostral and caudal hypothalamus and reduce heterogeneity of our tissues. While numerous studies have investigated how gestational exposure to EDCs alters reproductive physiology and gene expression in the brain in adulthood (Reviewed in (Dickerson et al, 2007)) we are the first to develop a comprehensive developmental profile (Postnatal day (P)15 – P90) of a suite of 48 neuroendocrine genes in these two brain regions that directly regulate GnRH synthesis and release in both males and females. We also have begun to identify potential epigenetic

mechanisms, namely DNA methylation, that may play roles in the programming of these long-term alterations caused by prenatal PCBs. Finally, we identify novel targets of EDCs in the hypothalamus, namely 2 clock genes *Arntl* and *Per2*, which are programmed in a male typical pattern in the female AVPV after exposure to estrogenic compounds. Taken together, these data not only highlight mechanisms of estrogenic EDCs but also provides us with novel information regarding the functional significance of the AVPV and ARC in male and female rats.

## MATERIALS AND METHODS

### Animals

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin Institutional Animal Care and Use Committee. Animals in this study were part of a larger study and were siblings of those discussed in a recent publication. For detailed methods on animal husbandry and the generation of individuals in this study see (Chapter 3). Briefly, Sprague-Dawley rats (3-4 month virgin females) were purchased from Harlan Laboratories, switched to low phytoestrogen Harlan-Teklad 2019 Global Diet ad libitum (Houston, TX) and impregnated in house. Rats were given at least 1 week to adjust to the new diet and environment before mating and were usually on the new diet for 2-3 weeks before conception. On embryonic day (E)16 and E18, (E1 = day after successful mating) dams were weighed and randomly assigned to one of 3 treatment groups and injected with 0.1ml vehicle (DMSO 99.5% Sigma #D4540); 50 $\mu$g/kg estradiol benzoate as a positive estrogenic control (EB, Sigma #E8515); or 1 mg/kg Aroclor 1221 (A1221; AccuStandard,

#C221N). Doses, routes and timing of exposure were chosen based on previous work in our laboratory (Dickerson et al., 2011a; Dickerson et al., 2011b; Steinberg et al., 2007; Steinberg et al., 2008) and others (Chung and Clemens, 1999; Gillette et al., 1987a; Gillette et al., 1987b; Murugesan et al., 2005a; Murugesan et al., 2005b). Based on previous literature (Takagi et al., 1986), we estimate that each pup is exposed to ~1:500 of the dose (i.e., 2 $\mu$g/kg A1221 or 100 ng of EB) which is within the range human exposure in utero (1 - 9 ppb ) (Karmaus and Zhu, 2004; Lackmann, 2002; Law et al., 2005; Longnecker et al., 2005; Schantz, 1996). Animals were housed under constant humidity and temperature (21-22°C) with a partially reversed 12:12 light cycle (lights on at 23:00). On the day after birth (postnatal day (P) 1), litter composition, birth weights and anogenital distance were recorded, and the litters were culled to equal sex ratios with a goal of 6 males and 6 females per litter. Body weights were monitored weekly and anogenital distance was measured weekly until weaning. Pups were weaned on P21 and housed with same sex littermates (2 -3 per cage). Rats were monitored for a secondary sex characteristic of the onset of puberty daily (preputial separation in males and vaginal opening in females). Following the onset of puberty, estrous cycles were monitored in by daily vaginal smears. Investigators were blind to treatment throughout the experiment. Because of the large number of animals necessary for both studies, animals were raised in 5 cohorts with treatments equally distributed across each cohort.

**Tissue Collection and Storage**

On P15, 30, 45 and 90 male and female littermates were euthanized approximately 1 – 3 hours before lights out via rapid decapitation, trunk bloods were collected and

endocrine tissues were removed and weighed. Those females who were postpubertal were euthanized on proestrus. Because not every litter had 6 males and 6 females each litter may not have been represented at each age which resulted in the final sample sizes varied for each age. Therefore we present a range of sample sizes for each treatment: DMSO: 14 - 20; EB: 15 - 21; A1221: 18-25. However, only a subset of 8 animals per group were used for gene expression. Brains were removed, sectioned and punches were collected using previously reported methods (Chapter 3). Trunk bloods were allowed to clot, and serum was separated via centrifugation (1500 X g for 5 min). Tissues and serum were stored at -80°C until use.

**Extraction of nucleic acids and preparation for PCR and pyrosequencing**

DNA and RNA were extracted from frozen AVPV and ARC punches of males and females using a Qiagen Allprep DNA/RNA mini kit according to manufacturer's protocols. RNA samples were eluted with RNase free water and treated with 1 U of TURBO® DNase (Applied Biosystems Inc., Foster City, CA) to rid samples of genomic DNA before ethanol precipitation. Resuspended samples were diluted to a concentration of 50 ng/$\mu$l based on concentrations obtained from both nanodrop and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) data which was also used to access purity and integrity. DNA samples obtained from Qiagen kits was diluted to 20ng/$\mu$l and 500ng of DNA were shipped to EpigenDX (Worcester, MA) for bisulfite conversion and pyrosequencing of *Per2* (2 assays: ADS3539 (-597 - -550bp from transcription start site (TSS)); ADS872 (-111 - +173bp from TSS) and *Ar* (1 assays: ADS070F (-70 - +39bp from TSS) regulatory regions.

**Taqman® Microfluidic Real-time PCR Cards**

RNA samples (200ng) were converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA) according to manufacturer's protocol and run on custom-designed microfluidic 48-gene PCR cards (Applied Biosystems Inc, Foster City, CA). Specific gene assays were chosen based on *a priori* hypotheses and published reports on their importance in neuroendocrine function and sensitivity to disruption by EDCs (46 genes of interest and 2 housekeeping genes; Tables 2.3 & 2.4).

Real-time PCR was carried out on an ABI ViiA7 using Taqman® Universal Mastermix (Applied Biosystems, Foster City CA) and the following run parameters: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression was determined for each sample using the comparative Ct Method (Livak and Schmittgen, 2001; Pfaffl, 2001; Schmittgen and Livak, 2008). Samples were normalized to 18s (the other housekeeping gene, Gapdh is sexually dimorphic in the AVPV) and calibrated to the median delta-Ct of the group with the lowest expression to determine fold-change in expression for each individual.

**Serum hormone assays**

Serum hormone concentrations were measured in the same radioimmunoassays as those presented in Walker et al., 2012 (Chapter 3). A brief description of the methods are included here. Serum LH was measured in duplicate 50 $\mu$l samples in the laboratory of Dr. Michael Woller, University of Wisconsin-Whitewater, by double antibody competitive binding RIA and intra-assay variability was 3.85%. Serum concentrations of testosterone

(MP Biomedicals) and estradiol (Beckman Coulter, Webster, TX) and were measured in 1 (intra-assay CV =1.397%) and 2 (intra-assay CV = 6.48% and 1.64%; inter-assay CV = 4.55%.) assays respectively. Sensitivity of the testosterone assay was 0.3ng/ml and estradiol was 2.2 pg/ml.

**Statistics**

Multiple regression analysis was conducted using PASW® software (IBM, Armonk NY) to compare each endpoint (genes, hormones, DNA methylation and endocrine tissues) using age, sex and treatment as independent variables. For gene expression data, statistics were performed using relative expression values for each sample. For those endpoints where a significant main effect of age, sex or treatment or an interaction was observed data were split by independent variables and follow-up analysis was performed to determine specific differences between each group. If data did not meet the assumptions for multiple regression analysis, data were transformed (natural log or square root) and reanalyzed. In a few cases, transformed data did not meet assumptions for statistical analysis by multiple regression. In those cases, data were analyzed using a Kruskal-Wallis test followed by Mann-Whitney test between each group. Because interactions can not be investigated using non-parametric testing, when a group effect was detected, data were split by significant variables and follow-up analysis was performed. If non-parametric testing was used for analysis independent variables are listed as Sex/Age/Treatment (Trt) rather than using an "X" to indicate interactions. For hormone concentrations an effect was considered significant at $p < 0.05$. For gene expression data a Benjamini and Hochberg False-Discovery Rate correction (Benjamini and Hochberg,

1995; Hochberg and Benjamini, 1990) was used to correct our p-values to account for the large number of variables measured. Parametric data were tested for outliers using the z-score of the residuals from the initial regression. A data point was considered an outlier if the residual was greater than 2.5 standard deviations from initial line of best fit. Non-parametric data were tested for outliers using the Grubbs' outlier test. Confirmed outliers were excluded from final analysis.

Vaginal smear data were analyzed using the same rule-based pattern matching techniques utilized previously (Chapter 3) to determine number and length of each cycle for each animal. Cycles were identified for each animal by scanning the smear data from the day of vaginal opening until euthanasia for the diestrus 2 to proestrus transition. The make-up and length of each cycle was recorded throughout the lifecycle. A repeated measures ANOVA was conducted using the moving average of the cycle length (using three cycle lengths) for each animal to account for any missing data. The output of this analysis was also used to investigate subtle differences in the cycles of each animal. This was accomplished by categorizing every unique cycle observed in each animals and comparing the categories of smears across different animals. This enabled us to look for series that were common to all animals and provide us with information regarding the composition of regular/irregular smears and determine if specific irregular series were more likely to occur in treated vs control females. Differences were analyzed using a Chi squared test and compared using the frequencies in control animals as our expected values.

Hierarchical cluster analysis was performed and heatmaps were generated using Multiple Experiment Viewer V4.8.1 (TM4.org) and clusters were validated using R statistical packages.

To determine if developmental gene expression profiles in females and males from each treatment were similar to control animals, gene expression data (sex X treatment) was compared using two methods, both implemented as Python scripts. The first method used Bayesian analysis to compare 2 developmental profiles (control vs treatment) and Bayes factors were computed as an indicator of the probability that gene expression data for two groups is generated by the same normal distribution (marginalized over mean and variance), versus the probability that the gene expression data is generated by two different normal distributions (marginalized over their parameters). A low (0-5), or negative, Bayes factor, expressed in decibans (dB), indicates no meaningful difference between the groups, whereas a high Bayes (over 20) factor indicates a high probability that each group's expression data was generated by a different distribution.

The second comparison method used an *ad hoc* measure of "distance" between the gene expression curves. A piecewise-linear curve for each group was generated using the average expression at each age, with ages along the x-axis and expression level along the y-axis. As a measure of "distance" between curves, the area between the curves was calculated using the geometric formulas for the area of a polygon.

Taken together, the 2 methods allowed us to compare the developmental profiles between the 2 sexes. While Bayes factors measure the probability that gene expression profiles are generated by different distributions, this method of analysis is exceptionally

sensitive to any one age group being different from each other. Therefore, in order to reduce the number of false positives, we used the area-between-curves as a way to confirm that the 2 sexes were similar or different as it is a measure of the actual difference between average gene expression levels. We assumed that a group was similar to the other if the area between the curves was reduced by 50% when compared to the control counterparts.

**Results**

Littermates exposed to A1221 *in utero* were monitored from birth through 90 days of age to determine if exposure to environmental endocrine disrupting chemicals alters hypothalamic development and reproductive function. Changes in endocrine tissues, female estrous cycles and timing of developmental transitions were monitored. To ascertain potential molecular mechanisms underlying the developmental effects of EDCs, expression of 48 genes were measured in 2 brain regions that are important for reproductive function: AVPV and ARC. We also investigated if developmental changes in DNA methylation of the *Ar* and *Per2* promoter regions were altered after exposure to EDCs in utero. In the AVPV and ARC, 28 and 12 genes, respectively, were changed by treatment (main effect or interaction $p < 0.05$). Of those affected by treatment, 18 genes in AVPV and 2 genes in the ARC survived a Benjamini and Hochberg False-Discovery Rate correction (Benjamini and Hochberg, 1995; Benjamini and Hochberg, 2000) and are indicated in bold in tables 2.3 and 2.4. However, because genes were specifically chosen based on *a priori* hypothesis we report significant effects as $p < 0.05$ and trends as $p < 0.1$.

**Effects of EDCs on somatic and reproductive development**

In males and females, treatment resulted in sex specific effects on the timing of eye opening (female only) and puberty (males only) (Table 2.1). Significant sex differences were detected for each endpoint. Therefore, follow-up analysis investigated sex specific effects of treatment using a 1-Way ANOVA for treatment. In males, treatment significantly delayed the timing of puberty as measured by preputial separation ($p = 0.017$) and post-hoc analysis revealed the effect to be specific to males exposed to A1221 compared to controls. In females, the timing of eye opening was delayed by treatment ($p = 0.027$). Post-hoc analysis revealed the effects were specific to females exposed to A1221. Treatment effects were also observed on estrous cycles. When compared to DMSO controls, females exposed to EB were less likely to be classified as having regular cycles (at least 70% of their cycles were regular) ($p = 0.008$); more likely to have an abnormal period of diestrus (6 or more days of L) ($p = 0.021$); more likely to transition from a leukocytic to cornified smear ($p = 0.003$) and have at least 2 elongated series of cornified and/or leukocytic smears (at least 5 days of C and/or L) ($p = 0.023$). Whereas, females exposed to A1221 were more likely to have at least 2 elongated series of cornified and/or leukocytic smears (at least 5 days of C and/or L) ($p = 0.096$) when compared to DMSO controls.

Treatment had sex and age specific effects on endocrine tissues and serum hormone concentrations. Males exposed to EB ($p = 0.122$) and A1221 ($p = 0.05$) had lower serum concentrations of LH on P15 and increased adrenal weights on P30 ($p = 0.116$; $p=0.037$). Males exposed to A1221 showed a trend for a decrease in gonadal

somatic index (GSI) ($p = 0.079$). Females exposed to EB showed a trend for lower serum estradiol on P15 ($p = 0.07$) and significantly higher adrenal weights on P30 ($p = 0.025$). No effects of A1221 were observed in females and no effects of treatment were observed in P45 or P90 in either males or females.

**Treatment alters gene expression in genes necessary for reproductive function in the female AVPV but not the ARC**

*Kiss1*, *Tac2*, *Esr1* and *Pdyn* are expressed in the same neurons in the ARC (Navarro et al., 2009) whereas, in the AVPV only *Kiss1* and *Esr1* are expressed in the same neurons (Mayer et al., 2010). Treatment resulted in age and sex specific effects on expression of all four genes in the AVPV (Figure 2.1 left panel) but not the ARC (Figure 2.1 right panel). To make the figure more legible, expression in the ARC was collapsed across treatments. For expression in individual treatments in the AVPV and ARC as well as p-values for all main effects and interactions see Tables 2.3 (AVPV) and 2.4 (ARC). In the AVPV, a significant effect of treatment (main effect or interaction) was observed for all 4 genes (*Kiss1*: Sex/Age/Trt $< 0.001$; *Tac2*: Sex/Age/Trt $< 0.001$; *Esr1*: Age X Trt: $p = 0.053$; *Pdyn*: Sex X Trt: $p = 0.012$). Further analysis revealed that most treatment effects were specific to females (*Kiss1*: age $p < 0.001$ and treatment $p = 0.046$; *Tac2*: age X treatment: $p = 0.021$; *Esr1* age X treatment: $p = 0.041$; *Pdyn* females age X treatment: $p = 0.017$; males: main treatment $p = 0.045$, expression in males exposed to EB is lower than DMSO and A1221 throughout development). T-tests were run between the sexes at each age to determine if expression was sexually dimorphic throughout development. *Kiss1* and *Esr1* were sexually dimorphic in the DMSO AVPV (*Kiss1*: P30 $p < 0.05$; *Esr1* P45 $p =$

0.058). Exposure to EB but not A1221 resulted in sexually dimorphic expression in *Kiss1* (P30, P45, P90 $p < 0.05$), *Tac2* (P90 $P < 0.05$) and *Pdyn* (P15 and P90 $P < 0.1$; P30 and P45 $p < 0.05$) with females having higher expression than males. In the ARC, no treatment effects were observed. However, main effects of sex and age were observed for *Kiss1* and *Tac2* ($p < 0.001$) and an interaction of sex and age was observed for *Tac2* ($p = 0.006$). A main effect of age was observed for *Esr1* ($p < 0.001$) along with a trend for an interaction of sex and age ($p = 0.071$). No main effects of age or age by sex interaction were observed for *Pdyn* but a trend for a main effect of sex was observed ($p = 0.085$).

***Gene expression profiles in the in the female AVPV are similar to males after gestational exposure to EDCs***

In the AVPV, 28 genes were altered by gestational exposure to EDCs (main effect or interaction of treatment $p < 0.05$) and are indicated in red on Figure 2.3 (A and B). Further analysis revealed that 21 of those genes were specifically affected in the female AVPV but not the male (*Pdyn* was affected in both males and females). These genes are highlighted in bold on Figure 2.3A. In order to determine if the genes affected by treatment exhibited a male-typical pattern of expression throughout development, expression pattern must have met the following criteria: 1) genes must display an age X treatment effect in the females; 2) gene expression in the treated females were determined to be from a distribution that was similar to DMSO males using Bayesian analysis; and 3) the area between the DMSO male and treated female curves was reduced by 50% when compared to the difference between the control males and females. Using these criteria, we were able to determine that 9 of the 21 genes altered in the female AVPV displayed a

male-typical expression pattern throughout development (Figure 2.2). These genes fell into 4 functional groups: steroid hormone receptors (A. *Ar*, B. *Thra*, & C. *Gper*), neuropeptides and their receptors (D. *Gal*, E. *Gnrh1*, & F. *Kiss1r*), epigenetic processes (G. *Dnmt1*) and circadian clock genes (H. *Arntl* & I. *Per2*).

In order to determine which genes shared similar expression patterns across development in each sex, hierarchical cluster analysis was conducted using the average linking method and correlation coefficients to express similarity. In the female AVPV, 2 large clusters were validated (indicated by a red box in Figure 2.3B). The top cluster includes genes that decrease from P15 and the bottom cluster is composed of genes that increase from P15 – P90. In males, there were very few validated clusters, but in general, the genes in the top clusters decrease from P15 – P90 or undergo very little change and the genes in the bottom cluster increase from P15 – P90. The genes significantly altered by treatment are indicated in red and those with specific effects in each sex are highlighted in bold. When comparing validated clusters in the male AVPV to the female we noted that the bottom cluster in the males consisted of 17 genes closely resembled a cluster in the female AVPV (indicated by a gray rectangle). These clusters shared 11 of 14 genes, 11 of which have a significant treatment effect (main effect or interaction) 5 of which had effects specific to females.

In the female and male ARC, 5 and 6 clusters were validated respectively, and many of these clusters had only a few genes (2 – 5). In general genes were groups by those that decreased from P15 – P90 (top) or increased from P15 – P90 (bottom). The genes significantly altered by treatment are indicated in red and those with specific effects in

each sex are highlighted in bold. When comparing validated clusters in the male ARC to the female we noted that the bottom cluster in the males consisted of 14 genes and closely resembled the lower cluster in the female ARC (indicated by a gray rectangle). These clusters shared 11 of 14 genes, 5 of which have a significant treatment effect (main effect or interaction).

**DNA methylation is not altered in *Ar* or *Per2* promoter throughout development**

In order to determine if epigenetic mechanisms were altered by exposure to EDCs we measured DNA methylation on 2 promoter regions of genes that were affected by treatment (*Ar* and *Per2*). These genes were chosen because each displayed a significant sex by age by treatment and had large CpG islands surrounding the transcription start sites (TSS). With the exception of two of CpGs the *Per2* promoter was almost completely demethylated by P15 and remained so throughout development (data not shown). The *Ar* promoter region (Figure 2.4) displayed sexually dimorphic methylation at each CpG investigated (-70bp - +39bp from the transcription start site) with females (Figure 2.4A) having higher methylation when compared to males (Figure 2.4B). This result was anticipated based on the location of *Ar* on the X chromosome. Further analysis revealed that one CpG at -63bp displayed a significant interaction of age and treatment in males ($p = 0.006$). While there were few changes in methylation we also investigated if methylation correlated with expression of *Ar* in individuals across all ages. Significant ($p < 0.05$) and trends ($0.05 < p < 0.1$) are discussed herein. In females exposed to EB and A1221 but not DMSO, expression was positively correlated with methylation at CpGs at -65bp (EB $p=0.087$), -51bp (EB $p = 0.014$ & A1221 $p = 0.044$), -13bp (EB $p = 0.06$), -5bp ($p = 0.09$)

and average methylation across the region (EB $p = 0.064$; A1221 $p = 0.085$). In males exposed to EB, expression was negatively correlated with methylation at CpG -63bp ($p = 0.037$). In males exposed to DMSO expression was positively correlated with methylation at -57bp ($p = 0.07$).

**Gene expression in the ARC is altered by treatment in males but not females**

In the ARC, 12 genes were altered by gestational exposure to EDCs (main effect or interaction of treatment $p < 0.05$) and are indicated in red on Figure 2.3 (C and D). Further analysis revealed that 6 of those genes were specifically affected in the male ARC but not the female (Figure 2.5). These genes fell into 2 general groups: sex steroid hormone receptors (A. *Ar*), neuropeptides and their receptors (B. *Lepr*, C. *Mc3r*, and D. *Avp*; E. *Oxt*), and neurotransmitter receptors (F. *Grin2d*). Additionally, there were 3 general expression patterns observed as a result of treatment. For *Ar* and *Lepr*, A1221 and EB increased expression at P45 and decreased at P90 (A1221 only). *Avp* and *Grin2d* expression was affected at P15 with *Avp* suppressed (although not significantly when compared to DMSO males) in the EB group, and *Grin2d* expression increased at P15 in the EB treated males. *Avp* was also suppressed at P30 in both A1221 and EB groups. Finally, *Oxt* and *Mc3r* had a significant effect of treatment ($p = 0.047$ and 0.036 respectively) with no age effects. For both genes, expression was increased throughout development by EB but not A1221.

## FIGURES AND TABLES

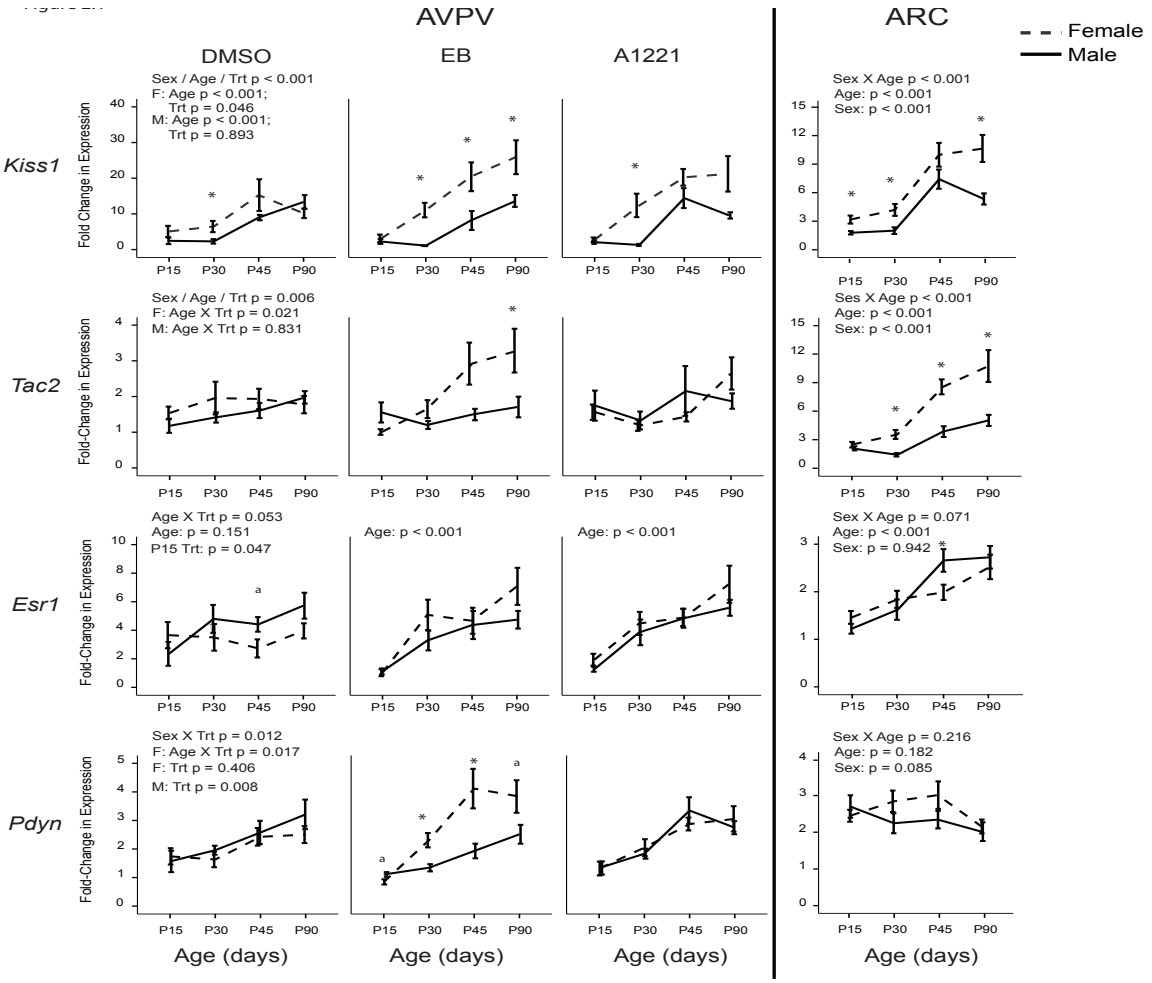



Figure 2.1: Expression profiles of genes required for reproduction in the AVPV & ARC.

Expression was characterized in females (dotted lines) and males (solid lines) from P15 – P90 in the AVPV (left) and ARC (right). Each of these genes had a significant effect of treatment or interaction of treatment in the AVPV but not ARC. Further analysis revealed that effects were specific to females in all genes except *Pdyn* where there effects in both males and females. Significant sex differences ($p < 0.05$) are indicated by an * and trends ($p < 0.1$) are indicated by a. In DMSO treated animals (first column), few sex differences are observed in the AVPV. In the EB but not A1221 treated animals, *Kiss1*, *Tac2* and *Pdyn* all had significant sex differences on P90 with other sex differences observed on P30 and P45 for Kiss1 and Pdyn. In the ARC, no significant effects of treatment were observed so data were collapsed across groups. For *Kiss1*, *Tac2* and *Esr1* significant sex and age effects were observed. For *Pdyn* a trend of sex differences were observed ($p = 0.085$).

Figure 2.2

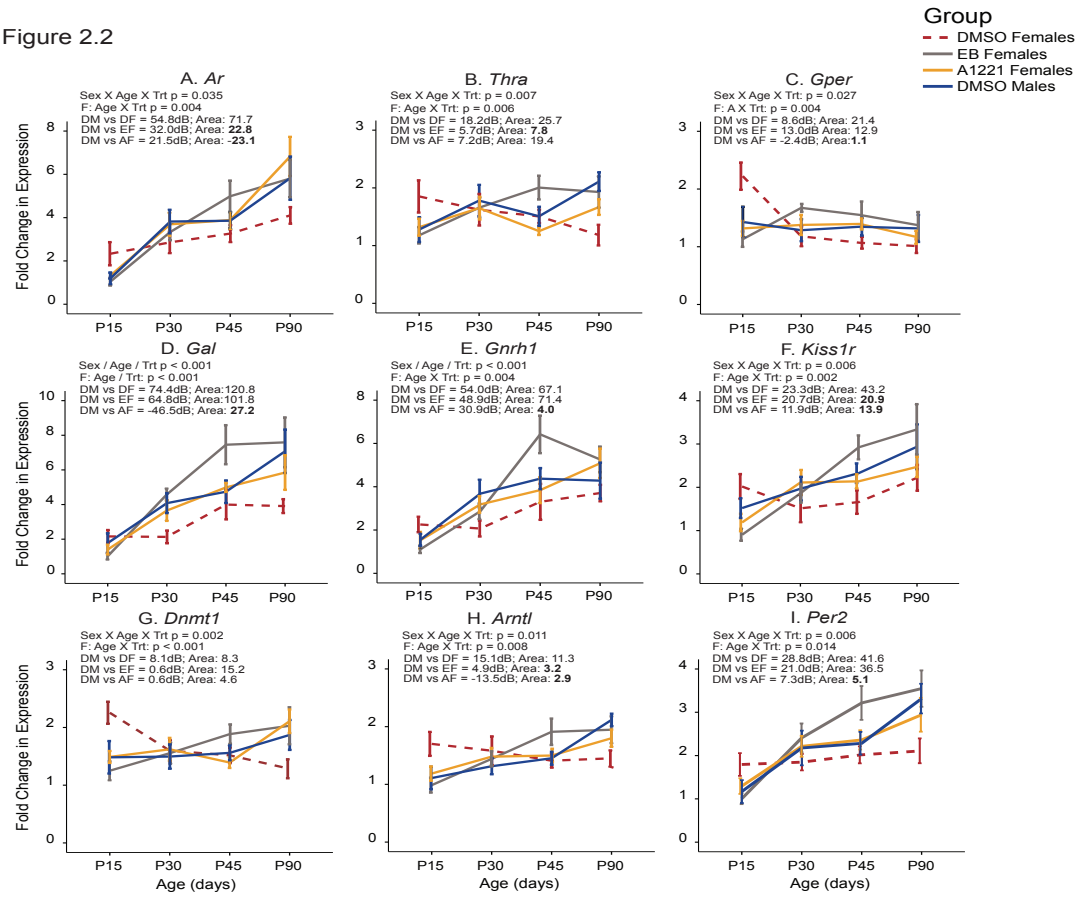



Figure 2.2: Expression profiles of genes affected by treatment in the female AVPV.

Data is only shown for DMSO males (DM; blue) and females (DF; red dotted) and EB females (EF; gray) and A1221 females (AF; yellow) because each gene was altered in the female but not males and had an age X treatment interaction ($p < 0.05$). In order to determine if the female profiles were similar to males or females, Bayes analysis was used to determine the probably of the two profiles coming from different distributions (indicated as dB). Additionally, area between the curves was calculated to determine if area was decreased in treated females were compared to the male curves. A gene was considered male typical if dB were decreased and the area between the curves was reduced by 50% when compared to when compared to the DMSO males and females (DM vs DF). Area in bold indicates a 50% reduction when compared to the DMSO curves. Of the 21 genes altered by treatment, only 9 met the criteria above. These genes fell 4 functional groups: steroid hormone receptors (*Ar* (A), *Thra* (B) & *Gper* (C)); neuropeptides and their receptors (*Gal* (D), *Gnrh1* (E) & *Kiss1r* (F)); epigenetic enzymes (*Dnmt1* (G)); circadian genes (*Arntl* (H) & *Per2* (I)).

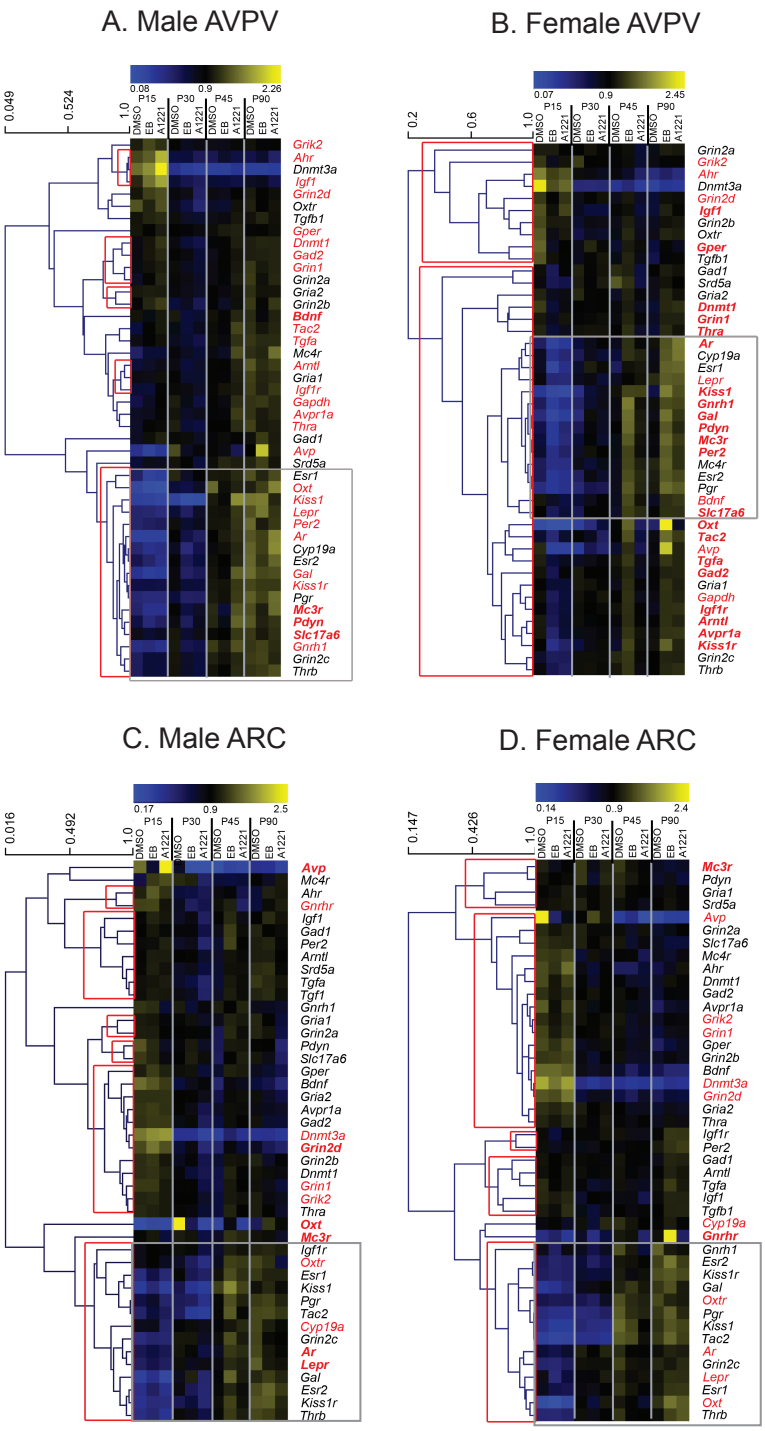



Figure 2.3: Clustergrams of gene expression.

Genes with a significant treatment (main effect of interaction) in males (A & C) and females (B & D) and the AVPV (A & B), ARC (C & D) are highlighted in red. Those with specific sex effects in each sex are indicated in bold text. Validated gene clusters are indicated by a red box. In males, only a few clusters were validated in the AVPV or ARC. Clusters highlighted in gray are similar between males and females.

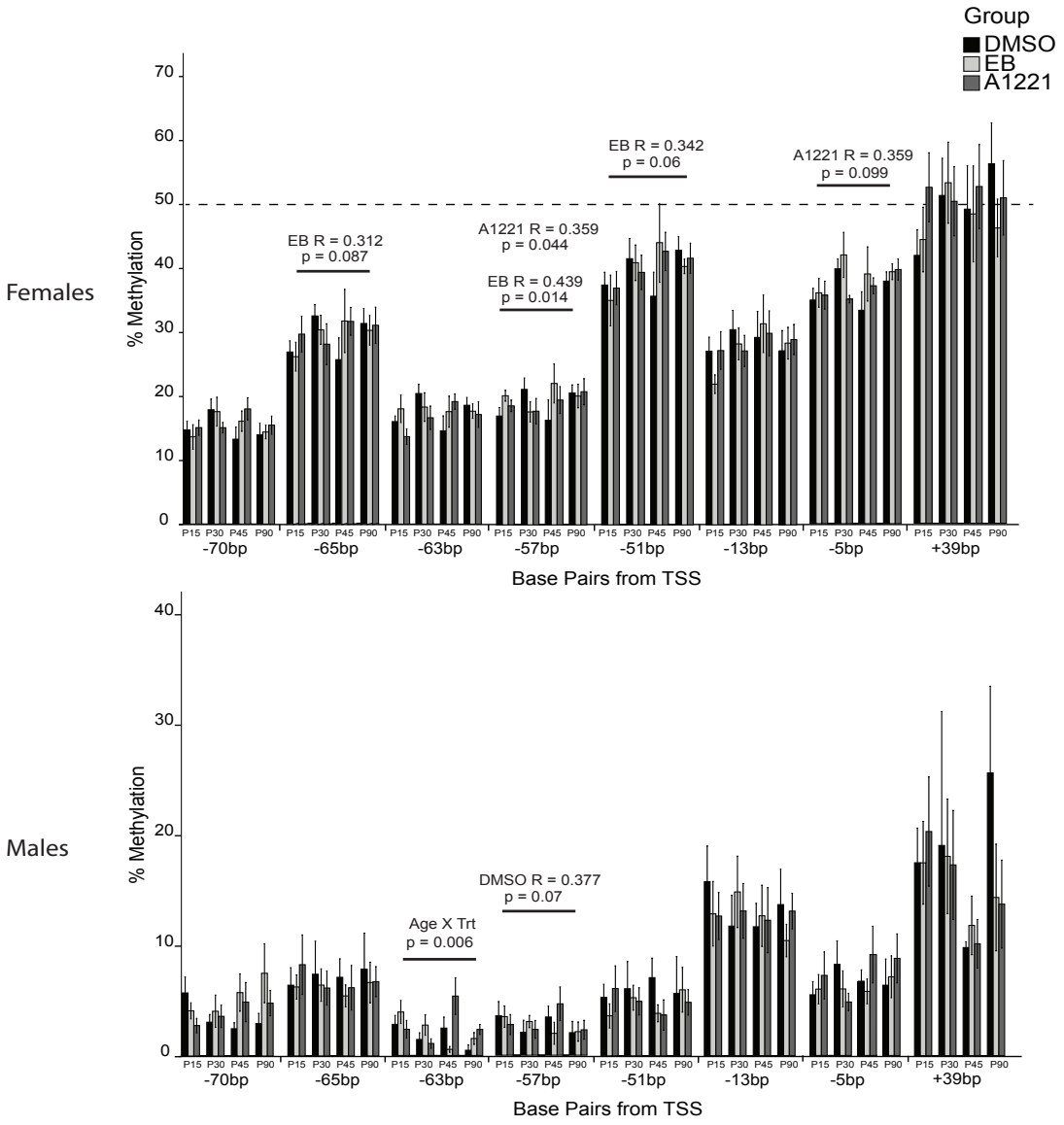



Figure 2.4: Methylation of individual CpG sites in the promoter region of *Ar*.

Percent methylation in females (top) and males (bottom) for each treatment group (DMSO = Black ; EB = light gray; A1221 = dark gray) at each age is shown. One site in the located at -63bp from the transcription start site (TSS) in the male promoter had a significant age X treatment interaction. There were significant sex differences at each CpG, with females having more methylation compared to males. Because Ar is on the X chromosome we were expected that methylation would be at 50% in the females as a reflection of X-inactivation (dotted line). Methylation was positively correlated with expression of Ar at the following CpGs in females: -65bp (EB), -57bp (EB & A1221), -51bp (EB), -5bp (A1221) and males: -57bp (DMSO).

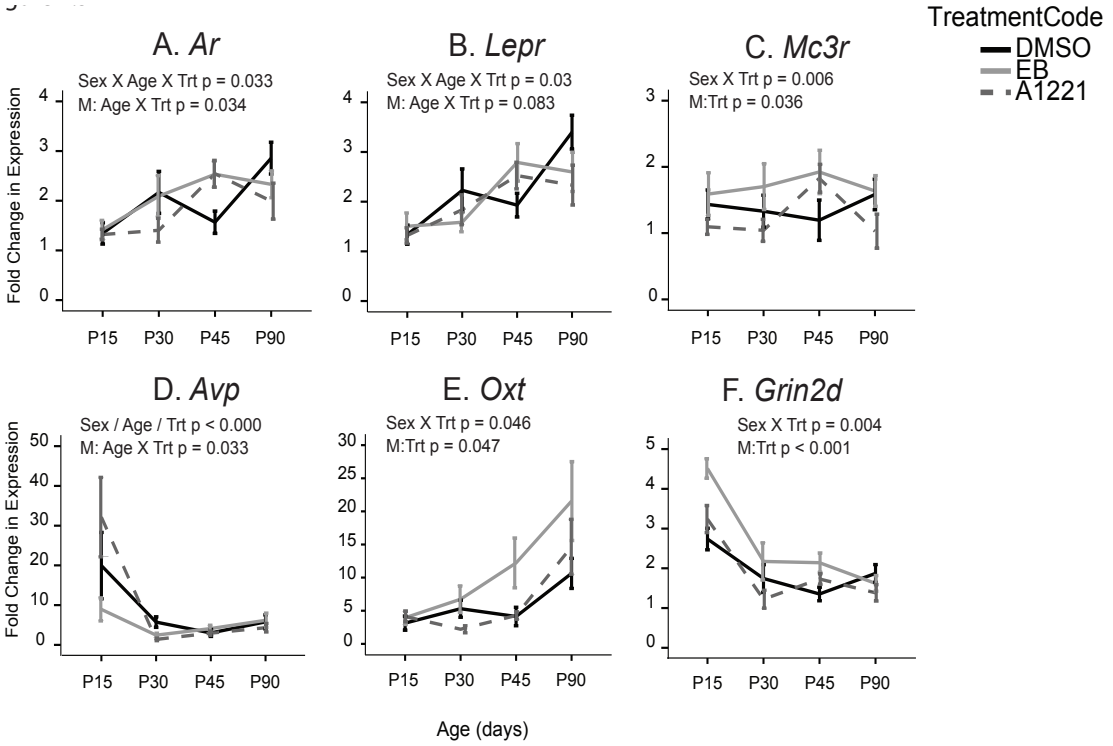



Figure 2.5: Expression of genes displaying an age X treatment interaction in the male ARC.

P-values obtained initial multiple regression analysis are listed at the top of each graph with male specific p-valued listed below. Two genes displayed a significant age X treatment interaction and 1 displayed a tread ($p<0.1$). These genes fell 3 functional groups: steroid hormone receptors (*Ar* (A); neuropeptides and their (*Lepr* (B), *Mc3r* (C), *Avp* (D) and *Oxt* (E)); and neurotransmitters and their receptors (*Grin2d* (F)). Three genes (*Mc3r*, *Oxt* and *Grin2d*) displayed a significant treatment effect ($p < 0.05$) with greater expression in the EB treated males. Three genes displayed a significant age X treatment interaction (*Ar*, *Lepr* & *Avp*) ($p < 0.05$). In DMSO males, *Ar* and *Lepr* expression was significantly increased on P90 when compared to P15 and P45. In EB males, *Lepr* is increased on P45 when compared to P15. In A1221 males, *Ar* expression is significantly lower on P15 when compared to P30 and P45 and *Lepr* expression is significantly lower on P15 when compared to P45 only.

| Endpoint | P-value F/M | DMSO Females | EB Females | A1221 Females | DMSO Males | EB Males | A1221 Males |
|---|---|---|---|---|---|---|---|
| P1 Weight | 0.401 / 0.293 | 6.08 (+/- 0.08) | 6.12 (+/- 0.08) | 6.17 (+/- 0.06) | 6.33 (+/- 0.09) | 6.45 (+/- 0.08) | 6.45 (+/- 0.06) |
| P1 AGD Ratio | 0.124 / 0.524 | 0.86 (+/- 0.01) | 0.90 (+/- 0.01) | 0.90 (+/- 0.02) | 1.89 (+/- 0.01) | 1.90 (+/- 0.02) | 1.91 (+/- 0.01) |
| P7 AGD Ratio | 0.976 / 0.275 | 1.20 (+/- 0.02) | 1.20 (+/- 0.01) | 1.20 (+/- 0.02) | 2.28 (+/- 0.02) | 2.33 (+/- 0.03) | 2.33 (+/- 0.02) |
| P14 AGD Ratio | 0.287 / 0.070 | 1.58 (+/- 0.01) | 1.55 (+/- 0.01) | 1.57 (+/- 0.01) | 2.73 (+/- 0.03) | 2.67 (+/- 0.02) | 2.77 (+/- 0.03) |
| Eye Opening | **0.027** / 0.321 | 15.19 (+/- 0.08) | 15.32 (+/- 0.08) | **15.48 (+/- 0.07)** | 15.36 (+/- 0.09) | 15.48 (+/- 0.08) | 15.54 (+/- 0.08) |
| Age Puberty | 0.470 / **0.017** | 34.08 (+/- 0.24) | 34.11 (+/- 0.25) | 34.45 (+/- 0.23) | 42.12 (+/- 0.2) | 42.51 (+/- 0.21) | **42.94 (+/- 0.2)** |
| Estrous Cycle Stats | | | | | | | |
| % Regular (P90 Females only) | | 85.0% | **63.2% p = 0.008** | 85.0% p = 1.0 | | | |
| % Animals with PD (All cycling Females) | | 5.1% | **13.5% p = 0.021** | 6.7% p = 0.64 | | | |
| Skip Proestrus (All cycling Females) | | 20.5% | **40.5% p = 0.003** | 28.9% p = 0.164 | | | |
| First 3 Cycles Regular (All cycling Females) | | 89.7% | 83.8% p = 0.232 | 84.4% p = 0.241 | | | |
| at least 2 strings of > 5 days of C/L | | 10.3% | **21.6% p = 0.023** | ***17.8%*** p = 0.096 | | | |

Table 2.1: Summary of EDC effects on developmental endpoints.

| Females | P15 | | | P30 | | |
|---|---|---|---|---|---|---|
| | DMSO | EB | A1221 | DMSO | EB | A1221 |
| T Concentration | 0.02 (+/- 0.003) | 0.11 (+/- 0.09) | 0.03 (+/- 0.01) | N/A | 0.11 (+/- 0.02) | N/A |
| LH Concentration | 1.93 (+/- 0.6) | 1.67 (+/- 0.6) | 2.85 (+/- 0.71) | 1.33 (+/- 0.16) | 1.35 (+/- 0.16) | 1.41 (+/- 0.14) |
| E2 Concentration | 93.43 (+/- 13.97) | **54.43 (+/- 8.53)** | 98.29 (+/- 16.1) | 7.09 (+/- 0.8) | 7.72 (+/- 1.63) | 7.06 (+/- 0.98) |
| Body Wt | 30.99 (+/- 1.11) | 33.44 (+/- 1.06) | 30.88 (+/- 0.76) | 82.64 (+/- 1.35) | 86.69 (+/- 1.86) | 80.74 (+/- 2.18) |
| GSI | 0.25 (+/- 0.01) | 0.24 (+/- 0.01) | 0.26 (+/- 0.01) | 0.39 (+/- 0.01) | 0.42 (+/- 0.03) | 0.42 (+/- 0.03) |
| Uterus Wt (mg) | 17.20 (+/- 0.8) | 18.01 (+/- 0.72) | 16.75 (+/- 0.73) | 62.13 (+/- 5.07) | 80.20 (+/- 17.72) | 62.14 (+/- 5.46) |
| Adrenal Wt (mg) | 0.78 (+/- 0.05) | 0.78 (+/- 0.04) | 0.70 (+/- 0.05) | 1.93 (+/- 0.1) | **2.34 (+/- 0.11)** | 2.19 (+/- 0.1) |
| Pit Wt (mg) | 2.19 (+/- 0.22) | 2.18 (+/- 0.1) | 2.02 (+/- 0.11) | 4.12 (+/- 0.19) | 4.45 (+/- 0.15) | 4.11 (+/- 0.22) |
| | | | | | | |
| **Males** | **P15** | | | **P30** | | |
| | DMSO | EB | A1221 | DMSO | EB | A1221 |
| T Concentration | 0.23 (+/- 0.1) | 0.02 (+/- 0.) | 0.12 (+/- 0.05) | 0.03 (+/- 0.01) | 0.02 (+/- 0.01) | 0.05 (+/- 0.02) |
| LH Concentration | 2.27 (+/- 0.46) | **1.10 (+/- 0.16)** | **1.08 (+/- 0.09)** | 1.35 (+/- 0.16) | 1.36 (+/- 0.13) | 1.79 (+/- 0.26) |
| E2 Concentration | 95.83 (+/- 15.32) | 102.01 (+/- 19.02) | 102.06 (+/- 20.62) | 8.50 (+/- 1.68) | 8.25 (+/- 1.08) | 9.96 (+/- 2.68) |
| Body Wt | 31.52 (+/- 1.07) | 34.21 (+/- 1.07) | 31.78 (+/- 1.01) | 91.97 (+/- 1.82) | 96.13 (+/- 3.04) | 93.08 (+/- 1.7) |
| GSI | 3.14 (+/- 0.09) | 3.10 (+/- 0.08) | 3.25 (+/- 0.08) | 9.54 (+/- 0.15) | 9.23 (+/- 0.14) | ***9.09 (+/- 0.13)*** |
| Adrenal Wt (mg) | 0.72 (+/- 0.08) | 0.80 (+/- 0.05) | 0.75 (+/- 0.04) | 1.97 (+/- 0.15) | **2.35 (+/- 0.07)** | **2.34 (+/- 0.21)** |
| Pit Wt (mg) | 1.86 (+/- 0.23) | 2.26 (+/- 0.23) | 1.78 (+/- 0.1) | 4.43 (+/- 0.24) | 4.54 (+/- 0.25) | 4.35 (+/- 0.23) |

| Females | P45 | | | P90 | | |
|---|---|---|---|---|---|---|
| | DMSO | EB | A1221 | DMSO | EB | A1221 |
| T Concentration | 0.02 (+/- 0.) | 0.03 (+/- 0.01) | 0.03 (+/- 0.01) | 0.02 (+/- 0.01) | 0.02 (+/- 0.) | 0.02 (+/- 0.01) |
| LH Concentration | 4.90 (+/- 2.61) | 1.55 (+/- 0.3) | 8.69 (+/- 5.29) | 4.17 (+/- 2.11) | 2.85 (+/- 1.65) | 4.61 (+/- 2.11) |
| E2 Concentration | 21.94 (+/- 2.41) | 20.36 (+/- 3.41) | 28.78 (+/- 4.09) | 20.15 (+/- 2.8) | 23.04 (+/- 4.55) | 20.16 (+/- 2.12) |
| Body Wt | 157.79 (+/- 2.23) | 156.91 (+/- 2.12) | 161.52 (+/- 3.01) | 262.95 (+/- 7.23) | 257.30 (+/- 5.19) | 249.41 (+/- 4.26) |
| GSI | 0.53 (+/- 0.01) | 0.54 (+/- 0.02) | 0.50 (+/- 0.02) | 0.46 (+/- 0.02) | 0.47 (+/- 0.01) | 0.46 (+/- 0.01) |
| Uterus Wt (mg) | 510.51 (+/- 33.97) | 469.03 (+/- 21.85) | 526.55 (+/- 31.09) | 666.70 (+/- 31.06) | 734.32 (+/- 52.13) | 727.10 (+/- 45.37) |
| Adrenal Wt (mg) | 3.64 (+/- 0.15) | 3.89 (+/- 0.23) | 3.96 (+/- 0.13) | 5.72 (+/- 0.14) | 5.20 (+/- 0.43) | 5.85 (+/- 0.18) |
| Pit Wt (mg) | 9.29 (+/- 0.32) | 8.53 (+/- 0.21) | 8.91 (+/- 0.36) | 13.84 (+/- 0.7) | 13.56 (+/- 0.44) | 13.42 (+/- 0.61) |
| | | | | | | |
| **Males** | **P45** | | | **P90** | | |
| | DMSO | EB | A1221 | DMSO | EB | A1221 |
| T Concentration | 0.20 (+/- 0.05) | 0.20 (+/- 0.04) | 0.34 (+/- 0.07) | 0.63 (+/- 0.12) | 0.57 (+/- 0.1) | 0.68 (+/- 0.16) |
| LH Concentration | 1.29 (+/- 0.14) | 1.14 (+/- 0.1) | 1.36 (+/- 0.11) | 0.72 (+/- 0.05) | 0.72 (+/- 0.07) | 0.87 (+/- 0.08) |
| E2 Concentration | 7.20 (+/- 0.6) | 7.84 (+/- 1.54) | 8.77 (+/- 0.75) | 7.86 (+/- 1.46) | 6.44 (+/- 0.78) | 8.11 (+/- 0.89) |
| Body Wt | 195.59 (+/- 3.98) | 197.52 (+/- 2.37) | 200.19 (+/- 3.39) | 384.60 (+/- 7.3) | 390.13 (+/- 8.35) | 383.46 (+/- 6.7) |
| GSI | 11.55 (+/- 0.62) | 12.03 (+/- 0.13) | 12.16 (+/- 0.14) | 9.62 (+/- 0.5) | 10.04 (+/- 0.19) | 10.18 (+/- 0.2) |
| Adrenal Wt (mg) | 3.14 (+/- 0.2) | 3.28 (+/- 0.08) | 3.18 (+/- 0.11) | 4.57 (+/- 0.17) | 4.83 (+/- 0.1) | 4.89 (+/- 0.12) |
| Pit Wt (mg) | 8.30 (+/- 0.23) | 8.69 (+/- 0.32) | 9.24 (+/- 0.38) | 12.56 (+/- 0.64) | 12.79 (+/- 0.27) | 12.40 (+/- 0.47) |

Table 2.2: Summary of EDC effects on serum hormone concentrations and endocrine tissues throughout development.

Table 2.3 EDC effects on gene expression in the AVPV from P15 – P90 in males and females.

| **Females Parametric Statistics** | | | | | | | | | DMSO | | | | EB | | | | A1221 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Gene Symbol** | **Gene Name** | **Sex** | **Age** | **Trt** | **Sex X Age** | **Sex X Trt** | **Age X Trt** | **Sex X Age X Trt** | **P15** | **P30** | **P45** | **P90** | **P15** | **P30** | **P45** | **P90** | **P15** | **P30** | **P45** | **P90** |
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.131 | **0.041** | 0.891 | 0.732 | 0.242 | 0.425 | 0.862 | 1.35 (+/-0.07) | 1.40 (+/-0.16) | 1.34 (+/-0.09) | 1.35 (+/-0.09) | 1.58 (+/-0.15) | 1.36 (+/-0.14) | 1.33 (+/-0.14) | 1.25 (+/-0.13) | 1.44 (+/-0.09) | 1.20 (+/-0.08) | 1.35 (+/-0.11) | 1.15 (+/-0.1) |
| *Ar* | androgen receptor | 0.727 | **0.000** | 0.502 | 0.235 | 0.839 | 0.030 | 0.035 | 2.33 (+/-0.54) | 2.86 (+/-0.5) | 3.26 (+/-0.39) | 4.10 (+/-0.39) | 1.04 (+/-0.18) | 3.34 (+/-0.38) | 4.99 (+/-0.73) | 5.81 (+/-0.86) | 1.32 (+/-0.15) | 3.69 (+/-0.53) | 3.89 (+/-0.38) | 6.83 (+/-0.91) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.285 | **0.000** | 0.846 | 0.050 | 0.345 | 0.260 | **0.011** | 1.70 (+/-0.21) | 1.58 (+/-0.25) | 1.41 (+/-0.12) | 1.45 (+/-0.14) | 0.99 (+/-0.13) | 1.44 (+/-0.13) | 1.91 (+/-0.23) | 1.95 (+/-0.23) | 1.19 (+/-0.12) | 1.48 (+/-0.15) | 1.50 (+/-0.12) | 1.80 (+/-0.15) |
| *Avpr1a* | arginine vasopressin receptor 1A | **0.002** | **0.000** | 0.468 | 0.672 | 0.478 | 0.128 | 0.024 | 2.05 (+/-0.29) | 1.65 (+/-0.22) | 1.60 (+/-0.24) | 1.92 (+/-0.22) | 1.03 (+/-0.15) | 1.67 (+/-0.2) | 2.43 (+/-0.34) | 2.42 (+/-0.31) | 1.42 (+/-0.13) | 1.74 (+/-0.17) | 2.00 (+/-0.2) | 2.45 (+/-0.32) |
| *Bdnf* | brain-derived neurotrophic factor | 0.106 | **0.007** | 0.795 | 0.604 | 0.015 | 0.192 | 0.150 | 1.67 (+/-0.28) | 1.15 (+/-0.23) | 1.29 (+/-0.25) | 1.27 (+/-0.14) | 1.24 (+/-0.29) | 1.32 (+/-0.16) | 2.20 (+/-0.32) | 1.94 (+/-0.22) | 1.10 (+/-0.14) | 1.45 (+/-0.21) | 1.64 (+/-0.1) | 2.02 (+/-0.27) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.743 | **0.000** | 0.408 | 0.627 | 0.120 | 0.219 | 0.652 | 2.37 (+/-0.57) | 1.85 (+/-0.34) | 2.13 (+/-0.64) | 2.46 (+/-0.19) | 1.20 (+/-0.34) | 2.43 (+/-0.48) | 3.70 (+/-0.79) | 4.40 (+/-0.67) | 1.49 (+/-0.21) | 2.18 (+/-0.37) | 3.37 (+/-0.52) | 5.00 (+/-1.11) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.979 | **0.003** | 0.785 | 0.198 | 0.696 | 0.084 | **0.002** | 2.25 (+/-0.19) | 1.60 (+/-0.18) | 1.52 (+/-0.14) | 1.28 (+/-0.17) | 1.25 (+/-0.16) | 1.55 (+/-0.18) | 1.88 (+/-0.17) | 2.03 (+/-0.32) | 1.49 (+/-0.1) | 1.62 (+/-0.2) | 1.39 (+/-0.09) | 2.11 (+/-0.2) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.110 | **0.000** | 0.814 | 0.117 | 0.699 | 0.608 | 0.278 | 9.43 (+/-2.09) | 1.90 (+/-0.31) | 1.39 (+/-0.16) | 1.03 (+/-0.14) | 5.59 (+/-0.9) | 1.85 (+/-0.22) | 1.90 (+/-0.21) | 1.37 (+/-0.23) | 6.19 (+/-0.57) | 1.92 (+/-0.21) | 1.20 (+/-0.09) | 1.29 (+/-0.13) |
| *Esr1* | estrogen receptor 1 | 0.726 | **0.000** | 0.284 | 0.600 | 0.191 | 0.053 | 0.530 | 3.66 (+/-0.92) | 3.50 (+/-0.93) | 2.73 (+/-0.64) | 3.96 (+/-0.53) | 1.02 (+/-0.25) | 5.08 (+/-1.06) | 4.66 (+/-0.91) | 7.07 (+/-1.3) | 1.94 (+/-0.42) | 4.48 (+/-0.81) | 4.89 (+/-0.61) | 7.22 (+/-1.3) |
| *Esr2* | estrogen receptor 2 | 0.067 | **0.000** | 0.922 | 0.461 | 0.084 | 0.178 | 0.094 | 1.91 (+/-0.36) | 1.81 (+/-0.14) | 2.58 (+/-0.35) | 3.41 (+/-0.45) | 1.06 (+/-0.18) | 2.72 (+/-0.39) | 4.42 (+/-0.83) | 4.66 (+/-0.63) | 1.23 (+/-0.1) | 2.39 (+/-0.27) | 3.71 (+/-0.3) | 4.26 (+/-0.58) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.438 | **0.000** | 0.985 | 0.457 | 0.694 | 0.501 | 0.029 | 1.80 (+/-0.28) | 1.84 (+/-0.38) | 1.69 (+/-0.19) | 1.56 (+/-0.32) | 1.10 (+/-0.17) | 1.72 (+/-0.18) | 2.24 (+/-0.24) | 2.21 (+/-0.32) | 1.30 (+/-0.25) | 1.89 (+/-0.25) | 1.48 (+/-0.13) | 2.07 (+/-0.18) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.828 | 0.120 | 0.927 | 0.199 | 0.540 | **0.003** | 0.027 | 2.22 (+/-0.24) | 1.18 (+/-0.17) | 1.07 (+/-0.1) | 1.01 (+/-0.12) | 1.13 (+/-0.13) | 1.67 (+/-0.07) | 1.55 (+/-0.23) | 1.37 (+/-0.23) | 1.32 (+/-0.13) | 1.38 (+/-0.18) | 1.40 (+/-0.1) | 1.16 (+/-0.11) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.417 | **0.000** | 0.909 | 0.084 | 0.216 | 0.580 | 0.212 | 1.44 (+/-0.16) | 1.63 (+/-0.31) | 1.27 (+/-0.06) | 1.38 (+/-0.18) | 1.13 (+/-0.17) | 1.56 (+/-0.05) | 1.86 (+/-0.23) | 1.90 (+/-0.25) | 1.13 (+/-0.08) | 1.45 (+/-0.14) | 1.38 (+/-0.1) | 1.70 (+/-0.12) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.569 | **0.023** | 0.471 | 0.199 | 0.431 | 0.437 | **0.011** | 1.54 (+/-0.17) | 1.30 (+/-0.19) | 1.27 (+/-0.1) | 1.15 (+/-0.15) | 1.16 (+/-0.13) | 1.40 (+/-0.12) | 1.67 (+/-0.15) | 1.62 (+/-0.2) | 1.50 (+/-0.19) | 1.44 (+/-0.13) | 1.28 (+/-0.05) | 1.61 (+/-0.11) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.622 | **0.000** | 0.546 | 0.290 | 0.684 | 0.817 | 0.075 | 2.30 (+/-0.35) | 1.25 (+/-0.14) | 1.39 (+/-0.16) | 1.36 (+/-0.17) | 1.80 (+/-0.3) | 1.44 (+/-0.17) | 1.85 (+/-0.17) | 1.70 (+/-0.22) | 1.82 (+/-0.14) | 1.40 (+/-0.15) | 1.32 (+/-0.1) | 1.77 (+/-0.19) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.410 | **0.000** | 0.460 | 0.068 | 0.727 | 0.721 | 0.085 | 1.32 (+/-0.19) | 1.69 (+/-0.22) | 2.00 (+/-0.18) | 1.58 (+/-0.23) | 1.01 (+/-0.16) | 1.70 (+/-0.17) | 2.17 (+/-0.13) | 1.97 (+/-0.2) | 1.04 (+/-0.18) | 1.67 (+/-0.15) | 1.65 (+/-0.14) | 1.91 (+/-0.18) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.260 | **0.000** | 0.749 | 0.189 | 0.798 | 0.611 | **0.017** | 2.32 (+/-0.29) | 1.51 (+/-0.26) | 1.49 (+/-0.21) | 1.34 (+/-0.23) | 1.70 (+/-0.21) | 1.72 (+/-0.17) | 1.93 (+/-0.16) | 1.82 (+/-0.28) | 2.26 (+/-0.26) | 1.83 (+/-0.17) | 1.39 (+/-0.08) | 1.77 (+/-0.18) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.345 | **0.000** | 0.857 | 0.353 | 0.382 | 0.113 | **0.031** | 1.38 (+/-0.17) | 1.51 (+/-0.18) | 1.55 (+/-0.12) | 1.24 (+/-0.17) | 0.99 (+/-0.12) | 1.43 (+/-0.13) | 1.87 (+/-0.12) | 1.88 (+/-0.16) | 1.11 (+/-0.14) | 1.49 (+/-0.12) | 1.40 (+/-0.08) | 1.90 (+/-0.15) |
| *Kiss1r* | KISS1 receptor | 0.148 | **0.000** | 0.755 | 0.626 | 0.587 | 0.133 | **0.006** | 2.02 (+/-0.29) | 1.51 (+/-0.32) | 1.66 (+/-0.27) | 2.22 (+/-0.3) | 0.90 (+/-0.14) | 1.86 (+/-0.22) | 2.92 (+/-0.28) | 3.34 (+/-0.58) | 1.19 (+/-0.2) | 2.11 (+/-0.29) | 2.14 (+/-0.16) | 2.47 (+/-0.24) |
| *Mc3r* | melanocortin 3 receptor | 0.282 | **0.000** | 0.839 | 0.952 | 0.010 | 0.268 | 0.100 | 2.77 (+/-0.59) | 2.84 (+/-0.72) | 3.35 (+/-0.76) | 3.46 (+/-0.51) | 1.27 (+/-0.32) | 3.57 (+/-0.25) | 5.84 (+/-0.93) | 6.20 (+/-1.08) | 1.85 (+/-0.39) | 2.97 (+/-0.44) | 4.02 (+/-0.45) | 5.08 (+/-0.88) |
| *Mc4r* | melanocortin 4 receptor | 0.096 | **0.000** | 0.457 | 0.141 | 0.657 | 0.731 | 0.168 | 1.58 (+/-0.22) | 2.19 (+/-0.52) | 2.13 (+/-0.39) | 3.00 (+/-0.32) | 1.12 (+/-0.12) | 1.92 (+/-0.29) | 3.56 (+/-0.82) | 3.99 (+/-0.7) | 1.14 (+/-0.13) | 2.21 (+/-0.33) | 2.92 (+/-0.28) | 3.22 (+/-0.46) |
| *Oxtr* | oxytocin receptor | 0.922 | **0.000** | 0.961 | 0.298 | 0.801 | 0.224 | 0.241 | 2.68 (+/-0.43) | 1.22 (+/-0.14) | 1.55 (+/-0.11) | 1.52 (+/-0.22) | 1.53 (+/-0.18) | 1.45 (+/-0.2) | 2.14 (+/-0.35) | 2.08 (+/-0.38) | 1.95 (+/-0.2) | 1.39 (+/-0.2) | 1.49 (+/-0.18) | 2.01 (+/-0.25) |
| *Pdyn* | prodynorphin | 0.197 | **0.000** | 0.402 | 0.495 | 0.012 | 0.121 | 0.056 | 1.74 (+/-0.29) | 1.63 (+/-0.27) | 2.43 (+/-0.31) | 2.50 (+/-0.3) | 0.85 (+/-0.09) | 2.31 (+/-0.25) | 4.11 (+/-0.69) | 3.84 (+/-0.57) | 1.32 (+/-0.24) | 2.05 (+/-0.3) | 2.87 (+/-0.23) | 3.05 (+/-0.44) |
| *Per2* | period homolog 2 | 0.248 | **0.000** | 0.636 | 0.913 | 0.006 | 0.448 | **0.006** | 1.79 (+/-0.26) | 1.85 (+/-0.19) | 2.02 (+/-0.2) | 2.11 (+/-0.29) | 1.00 (+/-0.13) | 2.41 (+/-0.33) | 3.22 (+/-0.39) | 3.55 (+/-0.42) | 1.30 (+/-0.18) | 2.21 (+/-0.24) | 2.36 (+/-0.22) | 2.94 (+/-0.39) |
| *Pgr* | progesterone receptor | 0.805 | **0.000** | 0.357 | 0.420 | 0.145 | 0.531 | 0.579 | 1.42 (+/-0.25) | 1.57 (+/-0.14) | 2.60 (+/-0.34) | 3.37 (+/-0.36) | 1.06 (+/-0.15) | 2.04 (+/-0.26) | 4.65 (+/-0.78) | 5.04 (+/-0.8) | 1.26 (+/-0.15) | 1.98 (+/-0.26) | 3.69 (+/-0.24) | 4.70 (+/-0.79) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.378 | **0.000** | 0.253 | 0.895 | 0.010 | 0.101 | 0.126 | 1.39 (+/-0.2) | 0.93 (+/-0.11) | 1.14 (+/-0.26) | 1.40 (+/-0.13) | 0.91 (+/-0.13) | 1.39 (+/-0.14) | 1.96 (+/-0.2) | 1.82 (+/-0.25) | 1.07 (+/-0.15) | 1.36 (+/-0.13) | 1.77 (+/-0.09) | 1.81 (+/-0.22) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.897 | **0.000** | 0.233 | 0.102 | 0.539 | 0.473 | 0.067 | 1.48 (+/-0.21) | 1.84 (+/-0.23) | 2.50 (+/-0.49) | 1.42 (+/-0.32) | 1.06 (+/-0.11) | 1.55 (+/-0.2) | 2.09 (+/-0.24) | 1.73 (+/-0.23) | 1.12 (+/-0.19) | 1.83 (+/-0.2) | 1.15 (+/-0.12) | 1.56 (+/-0.19) |
| *Tgfa* | transforming growth factor, alpha | 0.889 | **0.000** | 0.969 | 0.553 | 0.272 | 0.649 | **0.007** | 1.70 (+/-0.21) | 1.22 (+/-0.19) | 1.60 (+/-0.14) | 1.32 (+/-0.2) | 1.09 (+/-0.12) | 1.29 (+/-0.16) | 1.96 (+/-0.21) | 1.93 (+/-0.24) | 1.23 (+/-0.15) | 1.37 (+/-0.1) | 1.27 (+/-0.11) | 1.77 (+/-0.18) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.979 | 0.068 | 0.410 | 0.244 | 0.763 | 0.708 | 0.098 | 2.45 (+/-0.51) | 1.36 (+/-0.15) | 1.42 (+/-0.12) | 1.11 (+/-0.17) | 1.71 (+/-0.11) | 1.61 (+/-0.17) | 1.92 (+/-0.17) | 1.53 (+/-0.21) | 1.58 (+/-0.24) | 1.76 (+/-0.19) | 1.39 (+/-0.12) | 1.61 (+/-0.13) |
| *Thra* | thyroid hormone receptor, alpha | 0.835 | 0.087 | 0.728 | 0.124 | 0.162 | 0.419 | **0.001** | 1.85 (+/-0.28) | 1.62 (+/-0.27) | 1.51 (+/-0.12) | 1.18 (+/-0.18) | 1.18 (+/-0.14) | 1.66 (+/-0.17) | 2.00 (+/-0.21) | 1.93 (+/-0.27) | 1.32 (+/-0.15) | 1.65 (+/-0.2) | 1.25 (+/-0.07) | 1.67 (+/-0.14) |
| *Thrb* | thyroid hormone receptor, beta | 0.353 | **0.000** | 0.980 | 0.242 | 0.555 | 0.344 | 0.103 | 1.74 (+/-0.28) | 1.97 (+/-0.2) | 2.08 (+/-0.17) | 1.85 (+/-0.27) | 1.26 (+/-0.21) | 2.00 (+/-0.22) | 2.49 (+/-0.18) | 2.31 (+/-0.24) | 1.14 (+/-0.09) | 1.92 (+/-0.19) | 1.69 (+/-0.11) | 2.40 (+/-0.17) |
| **Females Nonparametric Statistics** | | | | | | | | | | | | | | | | | | | | |
| **Gene Symbol** | **Gene Name** | **Sex** | **Age** | **Trt** | **Sex/Age** | **Sex Trt** | **Age/Trt** | **Sex/Age /Trt** | **P15** | **P30** | **P45** | **P90** | **P15** | **P30** | **P45** | **P90** | **P15** | **P30** | **P45** | **P90** |
| *Ahr* | aryl hydrocarbon receptor | 0.410 | **0.000** | 0.432 | **0.000** | 0.693 | **0.000** | **0.000** | 4.13 (+/-0.66) | 2.35 (+/-0.42) | 2.10 (+/-0.39) | 1.08 (+/-0.19) | 3.16 (+/-0.35) | 2.07 (+/-0.24) | 1.83 (+/-0.19) | 1.32 (+/-0.22) | 3.43 (+/-0.6) | 1.92 (+/-0.2) | 1.31 (+/-0.13) | 1.08 (+/-0.11) |
| *Avp* | arginine vasopressin | 0.743 | **0.000** | 0.353 | **0.002** | 0.420 | **0.002** | **0.015** | 4.97 (+/-2.3) | 1.33 (+/-0.25) | 3.19 (+/-1.43) | 3.70 (+/-1.22) | 1.12 (+/-0.16) | 2.66 (+/-0.63) | 5.10 (+/-1.67) | 9.11 (+/-3.14) | 1.15 (+/-0.14) | 2.20 (+/-0.43) | 2.67 (+/-1.11) | 4.78 (+/-2.46) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.634 | 0.346 | 0.988 | 0.256 | 0.923 | 0.485 | 0.130 | 1.57 (+/-0.21) | 1.77 (+/-0.21) | 1.90 (+/-0.33) | 1.47 (+/-0.3) | 0.98 (+/-0.06) | 1.51 (+/-0.05) | 1.77 (+/-0.16) | 1.73 (+/-0.28) | 1.46 (+/-0.22) | 1.64 (+/-0.11) | 1.10 (+/-0.09) | 1.62 (+/-0.17) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.437 | **0.037** | 0.936 | 0.057 | 0.913 | 0.104 | 0.030 | 1.57 (+/-0.17) | 1.63 (+/-0.25) | 1.50 (+/-0.17) | 1.45 (+/-0.22) | 0.98 (+/-0.03) | 1.43 (+/-0.04) | 1.79 (+/-0.2) | 1.72 (+/-0.2) | 1.38 (+/-0.16) | 1.41 (+/-0.09) | 1.22 (+/-0.1) | 1.76 (+/-0.12) |
| *Gal* | galanin prepropeptide | 0.995 | **0.000** | 0.799 | **0.000** | 0.308 | **0.000** | **0.000** | 2.16 (+/-0.37) | 2.13 (+/-0.37) | 4.00 (+/-0.86) | 3.91 (+/-0.4) | 1.02 (+/-0.19) | 4.58 (+/-0.34) | 7.45 (+/-1.13) | 7.59 (+/-1.44) | 1.40 (+/-0.28) | 3.65 (+/-0.6) | 4.99 (+/-0.26) | 5.85 (+/-1.) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.790 | **0.000** | 0.944 | **0.000** | 0.711 | **0.000** | **0.000** | 2.26 (+/-0.36) | 2.07 (+/-0.37) | 3.30 (+/-0.83) | 3.71 (+/-0.38) | 1.11 (+/-0.17) | 2.87 (+/-0.36) | 6.42 (+/-0.87) | 5.26 (+/-0.6) | 1.53 (+/-0.39) | 3.18 (+/-0.38) | 3.84 (+/-0.6) | 5.08 (+/-0.68) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.938 | 0.079 | 0.856 | 0.112 | 0.832 | 0.375 | 0.057 | 1.56 (+/-0.2) | 1.60 (+/-0.37) | 1.07 (+/-0.05) | 1.13 (+/-0.12) | 1.11 (+/-0.13) | 1.30 (+/-0.12) | 1.54 (+/-0.16) | 1.59 (+/-0.24) | 1.18 (+/-0.06) | 1.28 (+/-0.09) | 1.21 (+/-0.09) | 1.38 (+/-0.08) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.942 | **0.003** | 0.630 | **0.002** | 0.808 | 0.081 | **0.019** | 1.78 (+/-0.25) | 1.82 (+/-0.28) | 1.27 (+/-0.09) | 1.21 (+/-0.17) | 1.15 (+/-0.04) | 1.30 (+/-0.07) | 1.37 (+/-0.11) | 1.27 (+/-0.14) | 1.45 (+/-0.12) | 1.32 (+/-0.07) | 1.05 (+/-0.11) | 1.33 (+/-0.11) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.484 | 0.323 | 0.697 | 0.509 | 0.559 | 0.790 | 0.553 | 1.56 (+/-0.2) | 1.33 (+/-0.13) | 1.36 (+/-0.18) | 1.22 (+/-0.18) | 1.56 (+/-0.39) | 1.48 (+/-0.17) | 1.43 (+/-0.12) | 1.58 (+/-0.23) | 1.35 (+/-0.1) | 1.36 (+/-0.13) | 1.06 (+/-0.18) | 1.52 (+/-0.12) |
| *Igf1* | insulin-like growth factor 1 | 0.016 | **0.000** | 0.617 | **0.000** | 0.227 | **0.000** | **0.000** | 2.61 (+/-0.62) | 1.47 (+/-0.13) | 1.76 (+/-0.17) | 1.34 (+/-0.16) | 1.83 (+/-0.11) | 1.29 (+/-0.09) | 1.82 (+/-0.21) | 2.05 (+/-0.36) | 2.46 (+/-0.57) | 1.28 (+/-0.06) | 1.31 (+/-0.09) | 1.75 (+/-0.18) |
| *Kiss1* | KiSS-1 metastasis-suppressor | **0.000** | **0.000** | 0.266 | **0.000** | **0.001** | **0.000** | **0.000** | 5.08 (+/-1.58) | 6.45 (+/-1.58) | 15.26 (+/-4.45) | 10.17 (+/-1.35) | 3.10 (+/-1.12) | 11.06 (+/-2.04) | 20.41 (+/-4.05) | 25.88 (+/-4.75) | 2.74 (+/-0.64) | 12.37 (+/-3.3) | 20.25 (+/-2.34) | 21.23 (+/-4.98) |
| *Lepr* | leptin receptor | 0.431 | **0.000** | 0.846 | **0.000** | 0.943 | **0.000** | **0.000** | 2.20 (+/-0.59) | 2.03 (+/-0.4) | 2.00 (+/-0.38) | 2.99 (+/-0.63) | 1.24 (+/-0.23) | 2.19 (+/-0.25) | 2.39 (+/-0.34) | 3.78 (+/-0.53) | 1.20 (+/-0.21) | 1.84 (+/-0.27) | 2.60 (+/-0.28) | 3.68 (+/-0.39) |
| *Oxt* | oxytocin, prepropeptide | 0.900 | **0.000** | 0.730 | **0.000** | 0.644 | 0.000 | **0.000** | 2.70 (+/-0.69) | 4.75 (+/-1.) | 15.98 (+/-6.6) | 9.20 (+/-1.79) | 1.15 (+/-0.29) | 7.69 (+/-1.55) | 26.01 (+/-8.7) | 42.06 (+/-15.71) | 1.17 (+/-0.26) | 11.97 (+/-2.38) | 9.82 (+/-1.58) | 13.23 (+/-2.18) |
| *Tac2* | tachykinin 2 | 0.100 | **0.000** | 0.903 | **0.000** | 0.605 | **0.002** | **0.006** | 1.54 (+/-0.18) | 1.96 (+/-0.46) | 1.93 (+/-0.29) | 1.77 (+/-0.24) | 1.01 (+/-0.08) | 1.65 (+/-0.25) | 2.92 (+/-0.59) | 3.29 (+/-0.61) | 1.56 (+/-0.22) | 1.19 (+/-0.16) | 1.43 (+/-0.13) | 2.65 (+/-0.45) |

Table 2.3, cont'd

| Males Parametric Statistics | | | | | | | | | DMSO | | | | EB | | | | A1221 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene Symbol | Gene Name | Sex | Age | Trt | Sex X Age | Sex X Trt | Age X Trt | Sex X Age X Trt | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 |
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.131 | **0.041** | 0.891 | 0.732 | 0.242 | 0.425 | 0.862 | 1.30 (+/-0.07) | 1.36 (+/-0.09) | 1.20 (+/-0.1) | 1.15 (+/-0.04) | 1.41 (+/-0.2) | 1.45 (+/-0.09) | 1.20 (+/-0.13) | 1.09 (+/-0.1) | 1.38 (+/-0.09) | 1.29 (+/-0.14) | 1.37 (+/-0.12) | 1.36 (+/-0.06) |
| *Ar* | androgen receptor | 0.727 | **0.000** | 0.502 | 0.235 | 0.839 | 0.030 | 0.035 | 1.19 (+/-0.26) | 3.82 (+/-0.55) | 3.86 (+/-0.4) | 5.82 (+/-1.) | 1.17 (+/-0.18) | 2.80 (+/-0.29) | 4.46 (+/-0.52) | 5.33 (+/-0.52) | 1.17 (+/-0.14) | 2.44 (+/-0.37) | 4.95 (+/-0.64) | 6.91 (+/-0.43) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.285 | **0.000** | 0.846 | 0.050 | 0.345 | 0.260 | **0.011** | 1.11 (+/-0.2) | 1.31 (+/-0.14) | 1.45 (+/-0.11) | 2.12 (+/-0.11) | 1.19 (+/-0.12) | 1.09 (+/-0.1) | 1.43 (+/-0.08) | 1.78 (+/-0.22) | 1.27 (+/-0.11) | 1.18 (+/-0.13) | 1.70 (+/-0.18) | 1.92 (+/-0.17) |
| *Avpr1a* | arginine vasopressin receptor 1A | **0.002** | **0.000** | 0.468 | 0.672 | 0.478 | 0.128 | 0.024 | 1.28 (+/-0.26) | 1.67 (+/-0.28) | 1.47 (+/-0.14) | 1.96 (+/-0.22) | 1.33 (+/-0.08) | 1.25 (+/-0.17) | 1.45 (+/-0.15) | 1.72 (+/-0.2) | 1.54 (+/-0.24) | 1.34 (+/-0.21) | 2.04 (+/-0.28) | 1.86 (+/-0.15) |
| *Bdnf* | brain-derived neurotrophic factor | 0.106 | **0.007** | 0.795 | 0.604 | 0.015 | 0.192 | 0.150 | 1.44 (+/-0.32) | 1.50 (+/-0.24) | 1.42 (+/-0.13) | 1.66 (+/-0.28) | 1.21 (+/-0.18) | 1.00 (+/-0.13) | 1.34 (+/-0.16) | 1.26 (+/-0.18) | 1.49 (+/-0.19) | 1.07 (+/-0.16) | 1.61 (+/-0.17) | 1.56 (+/-0.2) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.743 | **0.000** | 0.408 | 0.627 | 0.120 | 0.219 | 0.652 | 1.56 (+/-0.22) | 2.42 (+/-0.42) | 2.65 (+/-0.27) | 4.42 (+/-0.62) | 1.23 (+/-0.21) | 1.43 (+/-0.23) | 3.02 (+/-0.63) | 3.84 (+/-0.67) | 1.33 (+/-0.31) | 2.00 (+/-0.35) | 3.43 (+/-0.75) | 5.08 (+/-0.61) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.979 | **0.003** | 0.785 | 0.198 | 0.696 | 0.084 | **0.002** | 1.48 (+/-0.28) | 1.50 (+/-0.21) | 1.56 (+/-0.13) | 1.87 (+/-0.26) | 1.77 (+/-0.19) | 1.25 (+/-0.12) | 1.63 (+/-0.2) | 1.94 (+/-0.23) | 2.08 (+/-0.21) | 1.14 (+/-0.15) | 1.87 (+/-0.23) | 1.91 (+/-0.16) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.110 | **0.000** | 0.814 | 0.117 | 0.699 | 0.608 | 0.278 | 7.69 (+/-1.38) | 2.00 (+/-0.3) | 1.51 (+/-0.14) | 1.47 (+/-0.28) | 8.70 (+/-1.15) | 1.57 (+/-0.16) | 1.49 (+/-0.2) | 1.51 (+/-0.25) | 11.72 (+/-2.24) | 1.44 (+/-0.21) | 1.81 (+/-0.25) | 1.59 (+/-0.19) |
| *Esr1* | estrogen receptor 1 | 0.726 | **0.000** | 0.284 | 0.600 | 0.191 | 0.053 | 0.530 | 2.34 (+/-0.83) | 4.80 (+/-0.98) | 4.41 (+/-0.51) | 5.72 (+/-0.91) | 1.11 (+/-0.21) | 3.28 (+/-0.69) | 4.37 (+/-0.99) | 4.74 (+/-0.62) | 1.27 (+/-0.18) | 3.85 (+/-0.9) | 4.84 (+/-0.67) | 5.57 (+/-0.56) |
| *Esr2* | estrogen receptor 2 | 0.067 | **0.000** | 0.922 | 0.461 | 0.084 | 0.178 | 0.094 | 1.72 (+/-0.43) | 2.40 (+/-0.37) | 2.63 (+/-0.18) | 3.96 (+/-0.42) | 1.44 (+/-0.17) | 1.66 (+/-0.16) | 2.46 (+/-0.36) | 3.22 (+/-0.37) | 1.10 (+/-0.1) | 1.87 (+/-0.29) | 3.08 (+/-0.41) | 3.93 (+/-0.5) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.438 | **0.000** | 0.985 | 0.457 | 0.694 | 0.501 | 0.029 | 1.27 (+/-0.28) | 2.04 (+/-0.36) | 1.78 (+/-0.19) | 2.28 (+/-0.34) | 1.51 (+/-0.19) | 1.60 (+/-0.21) | 1.65 (+/-0.25) | 2.34 (+/-0.38) | 1.75 (+/-0.21) | 1.30 (+/-0.13) | 2.22 (+/-0.3) | 2.16 (+/-0.32) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.828 | 0.120 | 0.927 | 0.199 | 0.540 | **0.003** | 0.027 | 1.43 (+/-0.26) | 1.29 (+/-0.19) | 1.35 (+/-0.15) | 1.32 (+/-0.23) | 1.30 (+/-0.12) | 1.25 (+/-0.13) | 1.47 (+/-0.15) | 1.31 (+/-0.14) | 1.48 (+/-0.1) | 1.21 (+/-0.17) | 1.52 (+/-0.19) | 1.37 (+/-0.13) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.417 | **0.000** | 0.909 | 0.084 | 0.216 | 0.580 | 0.212 | 1.37 (+/-0.27) | 1.50 (+/-0.17) | 1.51 (+/-0.14) | 2.03 (+/-0.26) | 1.34 (+/-0.12) | 1.20 (+/-0.08) | 1.44 (+/-0.11) | 1.79 (+/-0.2) | 1.58 (+/-0.23) | 1.21 (+/-0.13) | 1.73 (+/-0.16) | 1.87 (+/-0.17) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.569 | **0.023** | 0.471 | 0.199 | 0.431 | 0.437 | **0.011** | 1.26 (+/-0.17) | 1.44 (+/-0.19) | 1.27 (+/-0.11) | 1.82 (+/-0.11) | 1.50 (+/-0.12) | 1.19 (+/-0.11) | 1.39 (+/-0.13) | 1.56 (+/-0.2) | 1.60 (+/-0.15) | 1.11 (+/-0.13) | 1.55 (+/-0.12) | 1.66 (+/-0.11) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.622 | **0.000** | 0.546 | 0.290 | 0.684 | 0.817 | 0.075 | 1.57 (+/-0.16) | 1.24 (+/-0.14) | 1.60 (+/-0.21) | 2.17 (+/-0.36) | 1.98 (+/-0.06) | 1.39 (+/-0.11) | 1.37 (+/-0.2) | 1.66 (+/-0.15) | 2.20 (+/-0.38) | 1.09 (+/-0.1) | 1.71 (+/-0.2) | 2.13 (+/-0.18) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.410 | **0.000** | 0.460 | 0.068 | 0.727 | 0.721 | 0.085 | 1.05 (+/-0.16) | 1.84 (+/-0.2) | 1.95 (+/-0.14) | 2.25 (+/-0.26) | 1.29 (+/-0.24) | 1.38 (+/-0.12) | 1.79 (+/-0.22) | 2.30 (+/-0.22) | 1.29 (+/-0.22) | 1.26 (+/-0.15) | 2.02 (+/-0.21) | 2.01 (+/-0.08) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.260 | **0.000** | 0.749 | 0.189 | 0.798 | 0.611 | **0.017** | 1.99 (+/-0.3) | 1.70 (+/-0.24) | 1.66 (+/-0.15) | 2.05 (+/-0.35) | 2.46 (+/-0.24) | 1.62 (+/-0.16) | 1.60 (+/-0.21) | 1.81 (+/-0.24) | 2.62 (+/-0.24) | 1.09 (+/-0.13) | 1.81 (+/-0.2) | 1.90 (+/-0.19) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.345 | **0.000** | 0.857 | 0.353 | 0.382 | 0.113 | **0.031** | 1.08 (+/-0.14) | 1.42 (+/-0.18) | 1.40 (+/-0.14) | 1.84 (+/-0.17) | 1.07 (+/-0.11) | 1.26 (+/-0.11) | 1.50 (+/-0.2) | 1.62 (+/-0.2) | 1.35 (+/-0.18) | 1.14 (+/-0.1) | 1.56 (+/-0.14) | 1.81 (+/-0.18) |
| *Kiss1r* | KISS1 receptor | 0.148 | **0.000** | 0.755 | 0.626 | 0.587 | 0.133 | **0.006** | 1.52 (+/-0.23) | 1.97 (+/-0.28) | 2.33 (+/-0.23) | 2.94 (+/-0.52) | 1.54 (+/-0.04) | 1.63 (+/-0.13) | 2.27 (+/-0.32) | 2.77 (+/-0.34) | 1.51 (+/-0.15) | 1.58 (+/-0.32) | 2.78 (+/-0.39) | 2.77 (+/-0.33) |
| *Mc3r* | melanocortin 3 receptor | 0.282 | **0.000** | 0.839 | 0.952 | 0.010 | 0.268 | 0.100 | 1.96 (+/-0.47) | 3.43 (+/-0.66) | 4.17 (+/-0.51) | 4.89 (+/-0.69) | 1.48 (+/-0.29) | 2.50 (+/-0.53) | 2.63 (+/-0.34) | 3.93 (+/-0.72) | 1.44 (+/-0.36) | 2.81 (+/-0.66) | 4.99 (+/-1.12) | 5.47 (+/-0.62) |
| *Mc4r* | melanocortin 4 receptor | 0.096 | **0.000** | 0.457 | 0.141 | 0.657 | 0.731 | 0.168 | 1.24 (+/-0.17) | 1.96 (+/-0.26) | 1.92 (+/-0.23) | 2.60 (+/-0.41) | 1.64 (+/-0.23) | 1.75 (+/-0.2) | 1.75 (+/-0.35) | 2.49 (+/-0.36) | 1.61 (+/-0.23) | 1.62 (+/-0.35) | 2.95 (+/-0.65) | 3.37 (+/-0.36) |
| *Oxtr* | oxytocin receptor | 0.922 | **0.000** | 0.961 | 0.298 | 0.801 | 0.224 | 0.241 | 2.55 (+/-0.52) | 1.49 (+/-0.22) | 1.43 (+/-0.15) | 2.03 (+/-0.45) | 2.05 (+/-0.13) | 1.27 (+/-0.14) | 1.40 (+/-0.13) | 1.71 (+/-0.21) | 2.52 (+/-0.28) | 0.96 (+/-0.1) | 1.85 (+/-0.3) | 1.89 (+/-0.22) |
| *Pdyn* | prodynorphin | 0.197 | **0.000** | 0.402 | 0.495 | 0.012 | 0.121 | 0.056 | 1.56 (+/-0.37) | 1.96 (+/-0.16) | 2.58 (+/-0.41) | 3.21 (+/-0.52) | 1.11 (+/-0.08) | 1.34 (+/-0.13) | 1.93 (+/-0.26) | 2.51 (+/-0.33) | 1.33 (+/-0.23) | 1.83 (+/-0.17) | 3.35 (+/-0.47) | 2.75 (+/-0.23) |
| *Per2* | period homolog 2 | 0.248 | **0.000** | 0.636 | 0.913 | 0.006 | 0.448 | **0.006** | 1.17 (+/-0.26) | 2.18 (+/-0.4) | 2.28 (+/-0.26) | 3.32 (+/-0.34) | 1.28 (+/-0.17) | 1.68 (+/-0.2) | 2.29 (+/-0.25) | 2.37 (+/-0.3) | 1.35 (+/-0.14) | 1.79 (+/-0.34) | 2.69 (+/-0.36) | 2.75 (+/-0.31) |
| *Pgr* | progesterone receptor | 0.805 | **0.000** | 0.357 | 0.420 | 0.145 | 0.531 | 0.579 | 1.52 (+/-0.25) | 2.12 (+/-0.35) | 3.15 (+/-0.35) | 4.29 (+/-0.8) | 1.39 (+/-0.15) | 1.66 (+/-0.16) | 2.95 (+/-0.36) | 3.78 (+/-0.43) | 1.41 (+/-0.11) | 1.71 (+/-0.27) | 3.71 (+/-0.45) | 4.49 (+/-0.4) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.378 | **0.000** | 0.253 | 0.895 | 0.010 | 0.101 | 0.126 | 1.27 (+/-0.21) | 1.58 (+/-0.21) | 1.47 (+/-0.09) | 1.90 (+/-0.23) | 1.14 (+/-0.17) | 1.14 (+/-0.14) | 1.41 (+/-0.11) | 1.60 (+/-0.19) | 1.22 (+/-0.17) | 1.28 (+/-0.21) | 1.89 (+/-0.14) | 1.81 (+/-0.12) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.897 | **0.000** | 0.233 | 0.102 | 0.539 | 0.473 | 0.067 | 1.20 (+/-0.18) | 1.67 (+/-0.25) | 1.60 (+/-0.14) | 1.83 (+/-0.25) | 1.37 (+/-0.12) | 1.50 (+/-0.24) | 1.86 (+/-0.38) | 2.09 (+/-0.36) | 1.32 (+/-0.12) | 1.17 (+/-0.1) | 1.77 (+/-0.23) | 1.68 (+/-0.22) |
| *Tgfa* | transforming growth factor, alpha | 0.889 | **0.000** | 0.969 | 0.553 | 0.272 | 0.649 | **0.007** | 1.28 (+/-0.21) | 1.24 (+/-0.15) | 1.50 (+/-0.16) | 1.86 (+/-0.23) | 1.40 (+/-0.2) | 1.13 (+/-0.11) | 1.34 (+/-0.2) | 1.75 (+/-0.21) | 1.53 (+/-0.19) | 1.02 (+/-0.09) | 1.83 (+/-0.27) | 1.77 (+/-0.18) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.979 | 0.068 | 0.410 | 0.244 | 0.763 | 0.708 | 0.098 | 1.70 (+/-0.32) | 1.63 (+/-0.28) | 1.41 (+/-0.08) | 1.69 (+/-0.25) | 1.88 (+/-0.23) | 1.30 (+/-0.05) | 1.45 (+/-0.18) | 1.87 (+/-0.29) | 1.95 (+/-0.17) | 1.26 (+/-0.15) | 1.73 (+/-0.21) | 1.67 (+/-0.16) |
| *Thra* | thyroid hormone receptor, alpha | 0.835 | 0.087 | 0.728 | 0.124 | 0.162 | 0.419 | **0.001** | 1.28 (+/-0.22) | 1.78 (+/-0.27) | 1.51 (+/-0.17) | 2.11 (+/-0.16) | 1.55 (+/-0.18) | 1.20 (+/-0.12) | 1.51 (+/-0.19) | 1.72 (+/-0.22) | 1.57 (+/-0.16) | 1.14 (+/-0.14) | 1.91 (+/-0.25) | 1.76 (+/-0.21) |
| *Thrb* | thyroid hormone receptor, beta | 0.353 | **0.000** | 0.980 | 0.242 | 0.555 | 0.344 | 0.103 | 1.16 (+/-0.18) | 1.97 (+/-0.32) | 1.96 (+/-0.18) | 2.34 (+/-0.39) | 1.41 (+/-0.19) | 1.49 (+/-0.11) | 1.82 (+/-0.23) | 2.42 (+/-0.33) | 1.37 (+/-0.15) | 1.36 (+/-0.12) | 2.23 (+/-0.23) | 2.62 (+/-0.23) |
| **Males Nonparametric Statistics** | | | | | | | | | | | | | | | | | | | | |
| Gene Symbol | Gene Name | Sex | Age | Trt | Sex/Age | Sex Trt | Age/Trt | Sex/Age /Trt | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 |
| *Ahr* | aryl hydrocarbon receptor | 0.410 | **0.000** | 0.432 | **0.000** | 0.693 | **0.000** | **0.000** | 3.35 (+/-0.39) | 1.79 (+/-0.27) | 1.62 (+/-0.14) | 1.70 (+/-0.35) | 3.70 (+/-0.36) | 1.83 (+/-0.25) | 1.96 (+/-0.36) | 1.93 (+/-0.41) | 4.92 (+/-0.8) | 1.58 (+/-0.17) | 1.75 (+/-0.23) | 1.42 (+/-0.14) |
| *Avp* | arginine vasopressin | 0.743 | **0.000** | 0.353 | **0.002** | 0.420 | **0.002** | **0.015** | 1.38 (+/-0.16) | 4.71 (+/-1.59) | 3.36 (+/-0.52) | 3.28 (+/-0.45) | 0.96 (+/-0.17) | 2.80 (+/-0.51) | 3.05 (+/-1.01) | 7.03 (+/-3.93) | 1.20 (+/-0.21) | 3.13 (+/-0.78) | 2.23 (+/-0.86) | 3.81 (+/-1.26) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.634 | 0.346 | 0.988 | 0.256 | 0.923 | 0.485 | 0.130 | 1.22 (+/-0.15) | 1.49 (+/-0.2) | 1.38 (+/-0.08) | 1.53 (+/-0.18) | 1.52 (+/-0.12) | 1.50 (+/-0.27) | 1.50 (+/-0.24) | 1.93 (+/-0.42) | 1.68 (+/-0.26) | 1.22 (+/-0.08) | 1.46 (+/-0.1) | 1.64 (+/-0.17) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.437 | **0.037** | 0.936 | 0.057 | 0.913 | 0.104 | 0.030 | 1.29 (+/-0.17) | 1.40 (+/-0.16) | 1.23 (+/-0.1) | 1.60 (+/-0.22) | 1.53 (+/-0.13) | 1.30 (+/-0.13) | 1.39 (+/-0.16) | 1.62 (+/-0.23) | 1.69 (+/-0.28) | 1.08 (+/-0.08) | 1.51 (+/-0.14) | 1.64 (+/-0.11) |
| *Gal* | galanin prepropeptide | 0.995 | **0.000** | 0.799 | **0.000** | 0.308 | **0.000** | **0.000** | 1.78 (+/-0.57) | 4.08 (+/-0.58) | 4.74 (+/-0.66) | 7.08 (+/-1.26) | 1.41 (+/-0.28) | 2.61 (+/-0.46) | 4.17 (+/-0.39) | 6.11 (+/-0.99) | 1.51 (+/-0.33) | 2.92 (+/-0.61) | 6.74 (+/-1.38) | 6.28 (+/-0.68) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.790 | **0.000** | 0.944 | **0.000** | 0.711 | **0.000** | **0.000** | 1.55 (+/-0.27) | 3.68 (+/-0.64) | 4.37 (+/-0.49) | 4.28 (+/-0.84) | 1.64 (+/-0.3) | 2.26 (+/-0.3) | 3.71 (+/-0.6) | 4.49 (+/-0.55) | 1.40 (+/-0.14) | 2.36 (+/-0.29) | 4.75 (+/-0.39) | 4.06 (+/-0.71) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.938 | 0.079 | 0.856 | 0.112 | 0.832 | 0.375 | 0.057 | 1.39 (+/-0.24) | 1.18 (+/-0.12) | 1.25 (+/-0.14) | 1.59 (+/-0.18) | 1.44 (+/-0.13) | 1.11 (+/-0.03) | 1.17 (+/-0.09) | 1.36 (+/-0.17) | 1.42 (+/-0.12) | 1.04 (+/-0.05) | 1.34 (+/-0.09) | 1.37 (+/-0.06) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.942 | **0.003** | 0.630 | **0.002** | 0.808 | 0.081 | **0.019** | 1.49 (+/-0.14) | 1.31 (+/-0.17) | 1.17 (+/-0.08) | 1.40 (+/-0.18) | 1.82 (+/-0.15) | 1.12 (+/-0.04) | 1.15 (+/-0.14) | 1.25 (+/-0.17) | 1.65 (+/-0.14) | 1.20 (+/-0.06) | 1.33 (+/-0.08) | 1.31 (+/-0.09) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.484 | 0.323 | 0.697 | 0.509 | 0.559 | 0.790 | 0.553 | 1.19 (+/-0.19) | 1.44 (+/-0.2) | 1.29 (+/-0.15) | 1.88 (+/-0.35) | 1.42 (+/-0.14) | 1.29 (+/-0.12) | 1.36 (+/-0.17) | 1.49 (+/-0.2) | 1.70 (+/-0.19) | 1.16 (+/-0.07) | 1.62 (+/-0.13) | 1.71 (+/-0.13) |
| *Igf1* | insulin-like growth factor 1 | 0.016 | **0.000** | 0.617 | **0.000** | 0.227 | **0.000** | **0.000** | 2.01 (+/-0.32) | 1.29 (+/-0.19) | 1.30 (+/-0.19) | 1.38 (+/-0.19) | 2.73 (+/-0.33) | 1.14 (+/-0.15) | 1.24 (+/-0.15) | 1.41 (+/-0.23) | 3.21 (+/-0.57) | 1.06 (+/-0.14) | 1.20 (+/-0.08) | 1.16 (+/-0.04) |
| *Kiss1* | KiSS-1 metastasis-suppressor | **0.000** | **0.000** | 0.266 | **0.000** | **0.001** | **0.000** | **0.000** | 2.45 (+/-0.92) | 2.30 (+/-0.64) | 8.97 (+/-0.77) | 13.33 (+/-1.94) | 2.25 (+/-0.68) | 1.08 (+/-0.11) | 8.14 (+/-2.65) | 13.62 (+/-1.66) | 2.07 (+/-0.47) | 1.29 (+/-0.3) | 14.42 (+/-2.79) | 9.57 (+/-0.89) |
| *Lepr* | leptin receptor | 0.431 | **0.000** | 0.846 | **0.000** | 0.943 | **0.000** | **0.000** | 1.49 (+/-0.22) | 2.03 (+/-0.13) | 2.85 (+/-0.52) | 3.79 (+/-0.59) | 1.44 (+/-0.19) | 1.61 (+/-0.12) | 2.80 (+/-0.35) | 4.40 (+/-0.55) | 1.29 (+/-0.17) | 2.16 (+/-0.25) | 3.44 (+/-0.1) | 3.37 (+/-0.41) |
| *Oxt* | oxytocin, prepropeptide | 0.900 | **0.000** | 0.730 | **0.000** | 0.644 | 0.000 | **0.000** | 2.85 (+/-0.65) | 8.88 (+/-3.22) | 18.23 (+/-3.91) | 13.40 (+/-3.29) | 1.51 (+/-0.39) | 8.30 (+/-2.34) | 11.98 (+/-2.66) | 15.56 (+/-4.16) | 0.99 (+/-0.33) | 9.60 (+/-2.86) | 13.46 (+/-3.52) | 20.15 (+/-5.17) |
| *Tac2* | tachykinin 2 | 0.100 | **0.000** | 0.903 | **0.000** | 0.605 | **0.002** | **0.006** | 1.18 (+/-0.2) | 1.41 (+/-0.14) | 1.61 (+/-0.21) | 1.98 (+/-0.18) | 1.55 (+/-0.28) | 1.20 (+/-0.11) | 1.49 (+/-0.16) | 1.71 (+/-0.29) | 1.74 (+/-0.42) | 1.33 (+/-0.25) | 2.15 (+/-0.7) | 1.87 (+/-0.22) |
| **Assays Not Expressed or Did Not Amplify** | | | | | | | | | | | | | | | | | | | | |
| *Dnmt3l* | DNA (cytosine-5-)-methyltransferase 3l | | | | | | | | | | | | | | | | | | | |
| *Gnrhr* | gonadotropin-releasing hormone receptor | | | | | | | | | | | | | | | | | | | |
| *Mc5r* | melanocortin5 receptor | | | | | | | | | | | | | | | | | | | |

EDC effects on gene expression in the AVPV from P15 – P90 in males and females. Bold indicates effects that survived false discovery rate correction.

Table 2.4 EDC effects on gene expression in the ARC from P15 – P90 in males and females.

| Female Parametric Statistics | | | | | | | | | DMSO | | | | EB | | | | A1221 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene Symbol | Gene Name | Sex | Age | Trt | Sex X Age | Sex X Trt | Age X Trt | Sex X Age X Trt | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 |
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.785 | 0.262 | 0.616 | 0.462 | 0.961 | 0.776 | 0.136 | 1.14 (+/-0.18) | 1.31 (+/-0.16) | 1.35 (+/-0.16) | 1.25 (+/-0.16) | 1.39 (+/-0.14) | 1.27 (+/-0.07) | 1.46 (+/-0.1) | 1.20 (+/-0.07) | 1.27 (+/-0.15) | 1.33 (+/-0.09) | 1.27 (+/-0.16) | 1.24 (+/-0.1) |
| *Ahr* | aryl hydrocarbon receptor | 0.467 | **0.000** | 0.353 | 0.252 | 0.056 | 0.951 | 0.991 | 2.45 (+/-0.25) | 1.72 (+/-0.35) | 1.26 (+/-0.24) | 1.30 (+/-0.27) | 2.27 (+/-0.37) | 1.35 (+/-0.23) | 1.26 (+/-0.22) | 1.69 (+/-0.45) | 3.02 (+/-0.51) | 1.83 (+/-0.2) | 1.62 (+/-0.23) | 2.08 (+/-0.45) |
| *Ar* | androgen receptor | 0.987 | **0.000** | 0.996 | 0.460 | 0.163 | 0.817 | 0.033 | 1.32 (+/-0.13) | 2.19 (+/-0.37) | 2.46 (+/-0.43) | 1.89 (+/-0.27) | 1.28 (+/-0.23) | 1.71 (+/-0.25) | 2.01 (+/-0.48) | 2.37 (+/-0.37) | 1.49 (+/-0.18) | 2.28 (+/-0.2) | 1.93 (+/-0.27) | 2.17 (+/-0.19) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.824 | 0.155 | 0.654 | 0.928 | 0.347 | 0.728 | 0.865 | 1.42 (+/-0.14) | 1.54 (+/-0.23) | 1.43 (+/-0.23) | 1.46 (+/-0.23) | 1.39 (+/-0.27) | 1.34 (+/-0.24) | 1.25 (+/-0.28) | 1.84 (+/-0.23) | 1.64 (+/-0.23) | 1.52 (+/-0.19) | 1.44 (+/-0.24) | 1.66 (+/-0.2) |
| *Avpr1a* | arginine vasopressin receptor 1A | 0.149 | **0.000** | 0.697 | 0.465 | 0.174 | 0.361 | 0.459 | 2.74 (+/-0.38) | 2.04 (+/-0.24) | 1.67 (+/-0.22) | 1.25 (+/-0.21) | 1.88 (+/-0.25) | 1.37 (+/-0.24) | 1.45 (+/-0.23) | 1.68 (+/-0.23) | 2.20 (+/-0.19) | 1.91 (+/-0.24) | 1.84 (+/-0.29) | 1.70 (+/-0.23) |
| *Bdnf* | brain-derived neurotrophic factor | 0.578 | **0.000** | 0.981 | 0.960 | 0.341 | 0.513 | 0.412 | 3.48 (+/-0.46) | 1.60 (+/-0.28) | 1.97 (+/-0.46) | 1.69 (+/-0.43) | 3.63 (+/-0.86) | 1.79 (+/-0.3) | 1.58 (+/-0.44) | 1.42 (+/-0.21) | 3.53 (+/-0.38) | 2.07 (+/-0.28) | 1.71 (+/-0.26) | 1.32 (+/-0.21) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.265 | 0.014 | 0.856 | 0.686 | 0.160 | 0.853 | 0.289 | 2.19 (+/-0.28) | 1.86 (+/-0.35) | 1.91 (+/-0.33) | 1.53 (+/-0.31) | 2.01 (+/-0.39) | 1.49 (+/-0.3) | 1.31 (+/-0.26) | 2.16 (+/-0.36) | 2.26 (+/-0.28) | 1.91 (+/-0.24) | 1.71 (+/-0.25) | 1.98 (+/-0.34) |
| *Esr1* | estrogen receptor 1 | 0.942 | **0.000** | 0.848 | 0.071 | 0.300 | 0.932 | 0.549 | 1.60 (+/-0.28) | 1.88 (+/-0.34) | 2.12 (+/-0.24) | 2.35 (+/-0.45) | 1.36 (+/-0.18) | 1.69 (+/-0.36) | 1.76 (+/-0.25) | 2.43 (+/-0.35) | 1.42 (+/-0.26) | 1.95 (+/-0.28) | 2.08 (+/-0.34) | 2.78 (+/-0.54) |
| *Esr2* | estrogen receptor 2 | 0.707 | **0.000** | 0.356 | 0.500 | 0.080 | 0.731 | 0.611 | 1.32 (+/-0.24) | 1.68 (+/-0.38) | 2.12 (+/-0.17) | 2.75 (+/-0.54) | 1.18 (+/-0.2) | 1.17 (+/-0.13) | 1.89 (+/-0.18) | 3.88 (+/-0.55) | 1.34 (+/-0.23) | 1.55 (+/-0.18) | 2.21 (+/-0.33) | 3.00 (+/-0.43) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.317 | 0.064 | 0.224 | 0.486 | 0.137 | 0.950 | 0.083 | 1.57 (+/-0.19) | 1.65 (+/-0.23) | 1.83 (+/-0.22) | 1.66 (+/-0.21) | 1.72 (+/-0.21) | 1.74 (+/-0.28) | 1.44 (+/-0.17) | 2.42 (+/-0.39) | 1.92 (+/-0.19) | 1.79 (+/-0.23) | 1.87 (+/-0.22) | 2.25 (+/-0.37) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.731 | **0.001** | 0.765 | 0.767 | 0.194 | 0.826 | 0.157 | 2.74 (+/-0.46) | 2.25 (+/-0.2) | 2.12 (+/-0.23) | 1.81 (+/-0.25) | 2.41 (+/-0.36) | 1.96 (+/-0.23) | 1.82 (+/-0.3) | 2.35 (+/-0.35) | 2.77 (+/-0.26) | 2.33 (+/-0.28) | 2.13 (+/-0.3) | 2.26 (+/-0.37) |
| *Gal* | galanin prepropeptide | 0.760 | **0.000** | 0.189 | 0.861 | 0.258 | 0.883 | 0.761 | 1.38 (+/-0.24) | 3.33 (+/-0.9) | 4.71 (+/-0.88) | 4.79 (+/-0.96) | 1.61 (+/-0.42) | 3.10 (+/-0.55) | 3.68 (+/-0.99) | 6.06 (+/-1.47) | 2.03 (+/-0.4) | 2.37 (+/-0.31) | 3.52 (+/-0.55) | 4.41 (+/-0.9) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.357 | 0.160 | 0.955 | 0.763 | 0.731 | 0.868 | 0.502 | 1.36 (+/-0.22) | 1.77 (+/-0.44) | 1.81 (+/-0.44) | 1.50 (+/-0.31) | 1.28 (+/-0.29) | 1.30 (+/-0.31) | 1.81 (+/-0.58) | 2.01 (+/-0.37) | 1.59 (+/-0.32) | 1.59 (+/-0.16) | 1.52 (+/-0.35) | 1.76 (+/-0.23) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.104 | 0.115 | 0.280 | 0.242 | 0.735 | 1.000 | 0.313 | 1.61 (+/-0.28) | 1.61 (+/-0.37) | 2.30 (+/-0.27) | 2.26 (+/-0.49) | 1.12 (+/-0.23) | 1.37 (+/-0.43) | 2.24 (+/-0.45) | 2.34 (+/-0.54) | 1.84 (+/-0.48) | 1.64 (+/-0.51) | 1.61 (+/-0.26) | 2.12 (+/-0.48) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.480 | 0.867 | 0.769 | 0.763 | 0.210 | 0.874 | 0.109 | 1.50 (+/-0.15) | 1.72 (+/-0.27) | 1.97 (+/-0.31) | 1.48 (+/-0.23) | 1.58 (+/-0.21) | 1.52 (+/-0.2) | 1.41 (+/-0.29) | 1.79 (+/-0.22) | 1.80 (+/-0.21) | 1.82 (+/-0.23) | 1.65 (+/-0.26) | 1.58 (+/-0.18) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.775 | **0.000** | 0.701 | 0.397 | 0.705 | 0.711 | 0.134 | 1.81 (+/-0.15) | 1.76 (+/-0.26) | 1.61 (+/-0.25) | 1.25 (+/-0.17) | 1.76 (+/-0.21) | 1.42 (+/-0.19) | 1.16 (+/-0.19) | 1.47 (+/-0.17) | 1.78 (+/-0.15) | 1.70 (+/-0.19) | 1.38 (+/-0.18) | 1.34 (+/-0.1) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.689 | **0.003** | 0.690 | 0.604 | 0.202 | 0.896 | 0.018 | 1.65 (+/-0.17) | 1.32 (+/-0.19) | 1.61 (+/-0.2) | 1.17 (+/-0.15) | 1.42 (+/-0.14) | 1.29 (+/-0.14) | 1.05 (+/-0.13) | 1.41 (+/-0.19) | 1.78 (+/-0.2) | 1.51 (+/-0.12) | 1.33 (+/-0.16) | 1.35 (+/-0.11) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.736 | **0.000** | 0.928 | 0.420 | 0.037 | 0.895 | 0.200 | 1.61 (+/-0.16) | 1.41 (+/-0.22) | 1.33 (+/-0.15) | 1.07 (+/-0.16) | 1.53 (+/-0.23) | 1.30 (+/-0.17) | 1.06 (+/-0.19) | 1.33 (+/-0.19) | 1.82 (+/-0.21) | 1.57 (+/-0.15) | 1.31 (+/-0.2) | 1.33 (+/-0.15) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.932 | 0.341 | 0.808 | 0.890 | 0.096 | 0.774 | 0.238 | 1.64 (+/-0.26) | 1.43 (+/-0.21) | 1.54 (+/-0.29) | 1.19 (+/-0.23) | 1.47 (+/-0.23) | 1.35 (+/-0.21) | 1.07 (+/-0.22) | 1.36 (+/-0.16) | 1.63 (+/-0.15) | 1.47 (+/-0.18) | 1.47 (+/-0.25) | 1.43 (+/-0.14) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.926 | **0.000** | 0.604 | 0.764 | 0.447 | 0.778 | 0.299 | 2.20 (+/-0.24) | 1.73 (+/-0.35) | 1.65 (+/-0.2) | 1.56 (+/-0.23) | 2.34 (+/-0.39) | 1.36 (+/-0.22) | 1.62 (+/-0.32) | 1.77 (+/-0.18) | 2.24 (+/-0.31) | 1.77 (+/-0.14) | 1.60 (+/-0.21) | 1.62 (+/-0.15) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.968 | **0.000** | 0.739 | 0.715 | 0.349 | 0.816 | 0.226 | 1.15 (+/-0.17) | 2.77 (+/-0.49) | 3.75 (+/-0.73) | 3.00 (+/-0.61) | 1.41 (+/-0.42) | 2.57 (+/-0.45) | 3.05 (+/-0.89) | 3.04 (+/-0.46) | 1.22 (+/-0.07) | 2.72 (+/-0.23) | 2.71 (+/-0.31) | 3.31 (+/-0.63) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.587 | **0.000** | 0.075 | 0.467 | 0.004 | 0.349 | 0.138 | 3.05 (+/-0.33) | 1.74 (+/-0.34) | 1.70 (+/-0.3) | 1.54 (+/-0.24) | 3.05 (+/-0.53) | 1.75 (+/-0.27) | 1.23 (+/-0.21) | 1.84 (+/-0.21) | 3.28 (+/-0.33) | 1.95 (+/-0.2) | 1.85 (+/-0.33) | 1.73 (+/-0.24) |
| *Igf1* | insulin-like growth factor 1 | 0.974 | 0.085 | 0.417 | 0.919 | 0.559 | 0.960 | 0.819 | 1.98 (+/-0.34) | 1.45 (+/-0.3) | 1.65 (+/-0.33) | 1.61 (+/-0.33) | 1.86 (+/-0.34) | 1.59 (+/-0.22) | 1.72 (+/-0.42) | 1.77 (+/-0.28) | 1.73 (+/-0.22) | 1.68 (+/-0.33) | 1.50 (+/-0.34) | 2.12 (+/-0.43) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.872 | **0.003** | 0.569 | 0.896 | 0.116 | 0.676 | 0.613 | 1.43 (+/-0.23) | 1.57 (+/-0.27) | 1.42 (+/-0.16) | 1.48 (+/-0.21) | 1.12 (+/-0.21) | 1.37 (+/-0.22) | 1.46 (+/-0.3) | 1.91 (+/-0.27) | 1.49 (+/-0.2) | 1.70 (+/-0.21) | 1.47 (+/-0.21) | 2.07 (+/-0.26) |
| *Kiss1* | KiSS-1 metastasis-suppressor | **0.000** | **0.000** | 0.337 | 0.276 | 0.592 | 0.971 | 0.729 | 3.58 (+/-0.86) | 4.77 (+/-1.66) | 13.74 (+/-2.96) | 10.34 (+/-2.5) | 2.87 (+/-0.59) | 3.86 (+/-0.77) | 8.12 (+/-1.54) | 13.17 (+/-2.77) | 3.14 (+/-0.83) | 4.04 (+/-0.7) | 8.14 (+/-0.98) | 8.45 (+/-2.05) |
| *Kiss1r* | KISS1 receptor | 0.086 | **0.000** | 0.635 | 0.577 | 0.130 | 0.944 | 0.689 | 1.41 (+/-0.28) | 1.98 (+/-0.49) | 2.33 (+/-0.38) | 3.20 (+/-0.89) | 1.34 (+/-0.28) | 1.67 (+/-0.31) | 1.88 (+/-0.25) | 3.91 (+/-0.67) | 1.42 (+/-0.12) | 1.61 (+/-0.18) | 2.62 (+/-0.29) | 3.45 (+/-0.44) |
| *Lepr* | leptin receptor | 0.821 | **0.000** | 0.616 | 0.495 | 0.207 | 0.642 | 0.030 | 1.74 (+/-0.23) | 2.00 (+/-0.34) | 2.28 (+/-0.28) | 2.24 (+/-0.27) | 1.04 (+/-0.12) | 1.85 (+/-0.23) | 1.84 (+/-0.25) | 2.90 (+/-0.44) | 1.61 (+/-0.23) | 2.46 (+/-0.31) | 2.30 (+/-0.34) | 2.76 (+/-0.31) |
| *Mc3r* | melanocortin 3 receptor | 0.824 | 0.277 | 0.413 | 0.408 | 0.006 | 0.808 | 0.410 | 1.59 (+/-0.26) | 1.98 (+/-0.32) | 1.76 (+/-0.22) | 1.30 (+/-0.25) | 1.22 (+/-0.23) | 1.24 (+/-0.21) | 1.38 (+/-0.32) | 1.24 (+/-0.14) | 1.41 (+/-0.18) | 1.75 (+/-0.23) | 1.50 (+/-0.27) | 1.27 (+/-0.16) |
| *Oxt* | oxytocin, prepropeptide | 0.459 | **0.000** | 0.335 | 0.025 | 0.046 | 0.396 | 0.200 | 1.38 (+/-0.19) | 5.99 (+/-1.98) | 11.88 (+/-4.5) | 7.49 (+/-1.86) | 1.36 (+/-0.29) | 3.60 (+/-0.99) | 6.69 (+/-2.79) | 17.80 (+/-4.61) | 2.79 (+/-1.04) | 7.94 (+/-1.63) | 10.72 (+/-4.18) | 15.89 (+/-4.79) |
| *Per2* | period homolog 2 | 0.949 | 0.016 | 0.710 | 0.389 | 0.070 | 0.896 | 0.569 | 1.90 (+/-0.4) | 1.86 (+/-0.4) | 1.97 (+/-0.36) | 2.22 (+/-0.29) | 1.64 (+/-0.26) | 1.71 (+/-0.34) | 1.61 (+/-0.36) | 2.35 (+/-0.29) | 1.83 (+/-0.33) | 1.99 (+/-0.31) | 2.02 (+/-0.4) | 2.84 (+/-0.54) |
| *Pgr* | progesterone receptor | **0.000** | **0.000** | 0.193 | 0.087 | 0.186 | 0.939 | 0.186 | 1.49 (+/-0.14) | 1.66 (+/-0.29) | 4.88 (+/-0.7) | 3.88 (+/-0.59) | 1.37 (+/-0.18) | 1.56 (+/-0.21) | 3.25 (+/-0.38) | 5.07 (+/-0.8) | 1.42 (+/-0.18) | 1.73 (+/-0.14) | 3.86 (+/-0.62) | 3.90 (+/-0.72) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.435 | 0.003 | 0.508 | 0.768 | 0.222 | 0.504 | 0.106 | 2.35 (+/-0.2) | 2.01 (+/-0.4) | 2.26 (+/-0.41) | 1.66 (+/-0.29) | 2.29 (+/-0.28) | 1.79 (+/-0.16) | 1.41 (+/-0.26) | 1.61 (+/-0.19) | 2.35 (+/-0.21) | 2.18 (+/-0.3) | 1.83 (+/-0.32) | 1.56 (+/-0.16) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.217 | 0.133 | 0.473 | 0.195 | 0.437 | 0.748 | 0.885 | 1.87 (+/-0.25) | 2.00 (+/-0.31) | 1.79 (+/-0.27) | 1.79 (+/-0.37) | 2.02 (+/-0.38) | 1.85 (+/-0.38) | 1.62 (+/-0.45) | 2.22 (+/-0.3) | 2.15 (+/-0.24) | 2.17 (+/-0.23) | 1.82 (+/-0.3) | 1.90 (+/-0.24) |
| *Tac2* | tachykinin 2 | **0.000** | **0.000** | 0.458 | 0.006 | 0.666 | 0.860 | 0.322 | 2.62 (+/-0.66) | 3.90 (+/-1.17) | 10.28 (+/-1.7) | 10.29 (+/-3.07) | 2.24 (+/-0.31) | 3.43 (+/-0.66) | 6.52 (+/-0.76) | 13.72 (+/-3.22) | 2.59 (+/-0.55) | 3.35 (+/-0.61) | 8.86 (+/-1.28) | 7.92 (+/-2.07) |
| *Tgfa* | transforming growth factor, alpha | 0.224 | **0.000** | 0.602 | 0.449 | 0.490 | 0.431 | 0.578 | 1.66 (+/-0.19) | 1.67 (+/-0.31) | 1.45 (+/-0.1) | 1.79 (+/-0.34) | 1.63 (+/-0.27) | 1.33 (+/-0.21) | 1.33 (+/-0.28) | 2.50 (+/-0.41) | 1.93 (+/-0.33) | 1.50 (+/-0.19) | 1.64 (+/-0.26) | 1.99 (+/-0.3) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.594 | 0.004 | 0.639 | 0.828 | 0.101 | 0.549 | 0.927 | 1.82 (+/-0.24) | 1.62 (+/-0.32) | 1.48 (+/-0.17) | 1.66 (+/-0.31) | 1.72 (+/-0.36) | 1.30 (+/-0.27) | 1.32 (+/-0.27) | 2.17 (+/-0.35) | 2.18 (+/-0.34) | 1.59 (+/-0.18) | 1.88 (+/-0.29) | 2.13 (+/-0.34) |
| *Thra* | thyroid hormone receptor, alpha | 0.510 | 0.286 | 0.922 | 0.836 | 0.247 | 0.882 | 0.471 | 1.68 (+/-0.24) | 1.68 (+/-0.35) | 1.61 (+/-0.29) | 1.30 (+/-0.24) | 1.59 (+/-0.33) | 1.37 (+/-0.26) | 1.18 (+/-0.29) | 1.68 (+/-0.25) | 1.87 (+/-0.26) | 1.72 (+/-0.23) | 1.53 (+/-0.31) | 1.43 (+/-0.24) |
| *Thrb* | thyroid hormone receptor, beta | 0.966 | **0.000** | 0.889 | 0.406 | 0.090 | 0.919 | 0.428 | 1.43 (+/-0.17) | 2.89 (+/-0.55) | 3.08 (+/-0.33) | 2.71 (+/-0.44) | 1.45 (+/-0.24) | 2.29 (+/-0.43) | 2.27 (+/-0.43) | 4.01 (+/-0.62) | 1.63 (+/-0.24) | 2.69 (+/-0.35) | 2.81 (+/-0.45) | 4.30 (+/-0.77) |
| **Female Nonparametric Statistics** | | | | | | | | | | | | | | | | | | | | |
| Gene Symbol | Gene Name | Sex | Age | Trt | Sex/Age | Sex Trt | Age/Trt | Sex/Age /Trt | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 |
| *Avp* | arginine vasopressin | 0.050 | 0.023 | 0.447 | **0.000** | 0.267 | 0.049 | **0.000** | 10.89 (+/-6.75) | 7.99 (+/-2.27) | 3.48 (+/-0.88) | 1.57 (+/-0.28) | 2.15 (+/-0.62) | 12.87 (+/-5.62) | 2.38 (+/-1.03) | 3.43 (+/-1.) | 3.91 (+/-1.15) | 6.40 (+/-1.41) | 1.99 (+/-0.51) | 2.27 (+/-0.59) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.212 | **0.001** | 0.592 | **0.004** | 0.359 | 0.031 | 0.012 | 1.14 (+/-0.1) | 1.60 (+/-0.21) | 1.99 (+/-0.38) | 1.57 (+/-0.17) | 1.47 (+/-0.35) | 1.11 (+/-0.11) | 1.79 (+/-0.39) | 1.80 (+/-0.18) | 1.28 (+/-0.12) | 1.89 (+/-0.21) | 1.49 (+/-0.19) | 1.53 (+/-0.29) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.802 | **0.000** | 0.994 | **0.000** | 0.995 | **0.000** | **0.000** | 6.49 (+/-0.81) | 1.78 (+/-0.42) | 1.25 (+/-0.21) | 1.18 (+/-0.21) | 5.76 (+/-1.34) | 1.36 (+/-0.27) | 1.54 (+/-0.45) | 1.72 (+/-0.29) | 5.61 (+/-0.71) | 1.50 (+/-0.12) | 1.54 (+/-0.3) | 1.54 (+/-0.2) |
| *Gnrhr* | gonadotropin-releasing hormone receptor | 0.15 | 0.035 | 0.109 | **0.002** | 0.214 | 0.109 | 0.03 | 0.64 (+/-0.3) | 0.93 (+/-0.09) | 1.60 (+/-0.32) | 1.54 (+/-0.5) | 0.53 (+/-0.12) | 0.71 (+/-0.24) | 1.13 (+/-0.21) | 3.94 (+/-1.14) | 0.55 (+/-0.12) | 1.09 (+/-0.29) | 1.57 (+/-0.77) | 0.94 (+/-0.29) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.611 | 0.001 | 0.560 | 0.011 | 0.517 | 0.051 | 0.159 | 2.38 (+/-0.33) | 1.60 (+/-0.36) | 1.56 (+/-0.22) | 1.11 (+/-0.17) | 2.19 (+/-0.4) | 1.56 (+/-0.35) | 1.63 (+/-0.36) | 1.43 (+/-0.19) | 2.17 (+/-0.27) | 1.87 (+/-0.29) | 1.69 (+/-0.34) | 1.59 (+/-0.23) |
| *Mc4r* | melanocortin 4 receptor | 0.092 | 0.034 | 0.802 | 0.074 | 0.622 | 0.065 | 0.223 | 1.57 (+/-0.26) | 1.36 (+/-0.22) | 1.85 (+/-0.46) | 1.55 (+/-0.25) | 1.68 (+/-0.3) | 1.78 (+/-0.35) | 1.36 (+/-0.32) | 1.56 (+/-0.29) | 2.56 (+/-0.65) | 1.67 (+/-0.45) | 0.91 (+/-0.07) | 1.67 (+/-0.13) |
| *Oxtr* | oxytocin receptor | 0.086 | **0.000** | 0.558 | **0.000** | 0.282 | **0.005** | 0.009 | 1.67 (+/-0.18) | 1.82 (+/-0.32) | 3.14 (+/-0.55) | 3.45 (+/-0.81) | 1.63 (+/-0.29) | 1.57 (+/-0.26) | 3.38 (+/-0.78) | 3.23 (+/-0.45) | 1.76 (+/-0.32) | 2.15 (+/-0.18) | 3.16 (+/-0.69) | 3.01 (+/-0.71) |
| *Pdyn* | prodynorphin | 0.085 | 0.182 | 0.517 | 0.216 | 0.129 | 0.386 | 0.107 | 2.53 (+/-0.3) | 3.44 (+/-0.6) | 3.26 (+/-0.57) | 2.08 (+/-0.37) | 2.35 (+/-0.38) | 1.96 (+/-0.31) | 3.12 (+/-0.84) | 2.41 (+/-0.32) | 2.52 (+/-0.19) | 3.20 (+/-0.49) | 2.73 (+/-0.61) | 2.02 (+/-0.33) |

Table 2.4, cont.

| Male Parametric Statistics | | | | | | | | | DMSO | | | | EB | | | | A1221 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene Symbol | Gene Name | Sex | Age | Trt | Sex X Age | Sex X Trt | Age X Trt | Sex X Age X Trt | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 |
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.785 | 0.262 | 0.616 | 0.462 | 0.961 | 0.776 | 0.136 | 1.37 (+/-0.08) | 1.24 (+/-0.11) | 1.42 (+/-0.1) | 1.05 (+/-0.12) | 1.32 (+/-0.13) | 1.55 (+/-0.13) | 1.05 (+/-0.13) | 1.30 (+/-0.07) | 1.26 (+/-0.11) | 1.35 (+/-0.08) | 1.24 (+/-0.06) | 1.18 (+/-0.14) |
| *Ahr* | aryl hydrocarbon receptor | 0.467 | **0.000** | 0.353 | 0.252 | 0.056 | 0.951 | 0.991 | 2.20 (+/-0.31) | 1.51 (+/-0.31) | 1.62 (+/-0.4) | 1.65 (+/-0.18) | 2.96 (+/-0.47) | 1.57 (+/-0.35) | 1.79 (+/-0.26) | 2.48 (+/-0.62) | 2.43 (+/-0.1) | 1.30 (+/-0.3) | 1.43 (+/-0.24) | 2.02 (+/-0.35) |
| *Ar* | androgen receptor | 0.987 | **0.000** | 0.996 | 0.460 | 0.163 | 0.817 | 0.033 | 1.34 (+/-0.22) | 2.16 (+/-0.42) | 1.56 (+/-0.23) | 2.85 (+/-0.32) | 1.41 (+/-0.19) | 2.09 (+/-0.42) | 2.53 (+/-0.26) | 2.33 (+/-0.27) | 1.32 (+/-0.11) | 1.40 (+/-0.24) | 2.54 (+/-0.27) | 1.99 (+/-0.36) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.824 | 0.155 | 0.654 | 0.928 | 0.347 | 0.728 | 0.865 | 1.41 (+/-0.25) | 1.42 (+/-0.23) | 1.14 (+/-0.13) | 1.64 (+/-0.21) | 1.60 (+/-0.24) | 1.52 (+/-0.28) | 1.49 (+/-0.21) | 1.82 (+/-0.2) | 1.60 (+/-0.14) | 1.13 (+/-0.21) | 1.50 (+/-0.15) | 1.42 (+/-0.22) |
| *Avpr1a* | arginine vasopressin receptor 1A | 0.149 | **0.000** | 0.697 | 0.465 | 0.174 | 0.361 | 0.459 | 2.61 (+/-0.39) | 1.59 (+/-0.24) | 1.60 (+/-0.14) | 1.87 (+/-0.29) | 2.34 (+/-0.34) | 1.92 (+/-0.28) | 2.08 (+/-0.33) | 1.87 (+/-0.27) | 2.54 (+/-0.22) | 1.37 (+/-0.26) | 2.13 (+/-0.26) | 1.72 (+/-0.31) |
| *Bdnf* | brain-derived neurotrophic factor | 0.578 | **0.000** | 0.981 | 0.960 | 0.341 | 0.513 | 0.412 | 3.77 (+/-0.61) | 1.74 (+/-0.33) | 1.07 (+/-0.12) | 1.73 (+/-0.34) | 3.39 (+/-0.45) | 2.13 (+/-0.38) | 1.91 (+/-0.36) | 1.79 (+/-0.54) | 3.11 (+/-0.52) | 1.21 (+/-0.22) | 2.01 (+/-0.23) | 1.23 (+/-0.21) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.265 | 0.014 | 0.856 | 0.686 | 0.160 | 0.853 | 0.289 | 2.43 (+/-0.37) | 1.86 (+/-0.38) | 1.37 (+/-0.18) | 2.15 (+/-0.25) | 2.50 (+/-0.46) | 1.68 (+/-0.39) | 2.53 (+/-0.34) | 2.20 (+/-0.3) | 2.21 (+/-0.11) | 1.36 (+/-0.3) | 1.93 (+/-0.28) | 1.87 (+/-0.39) |
| *Esr1* | estrogen receptor 1 | 0.942 | **0.000** | 0.848 | 0.071 | 0.300 | 0.932 | 0.549 | 1.08 (+/-0.18) | 2.11 (+/-0.49) | 2.09 (+/-0.25) | 2.93 (+/-0.34) | 1.47 (+/-0.21) | 1.53 (+/-0.31) | 3.14 (+/-0.46) | 2.68 (+/-0.39) | 1.11 (+/-0.14) | 1.23 (+/-0.24) | 2.75 (+/-0.45) | 2.56 (+/-0.53) |
| *Esr2* | estrogen receptor 2 | 0.707 | **0.000** | 0.356 | 0.500 | 0.080 | 0.731 | 0.611 | 1.04 (+/-0.11) | 1.46 (+/-0.24) | 2.16 (+/-0.23) | 3.24 (+/-0.33) | 1.35 (+/-0.17) | 1.61 (+/-0.29) | 2.77 (+/-0.41) | 3.88 (+/-0.39) | 1.06 (+/-0.14) | 0.96 (+/-0.11) | 2.35 (+/-0.38) | 2.89 (+/-0.34) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.317 | 0.064 | 0.224 | 0.486 | 0.137 | 0.950 | 0.083 | 1.75 (+/-0.23) | 1.79 (+/-0.32) | 1.57 (+/-0.19) | 2.28 (+/-0.23) | 2.02 (+/-0.26) | 1.91 (+/-0.36) | 2.68 (+/-0.32) | 2.04 (+/-0.3) | 1.84 (+/-0.16) | 1.33 (+/-0.26) | 2.02 (+/-0.29) | 1.93 (+/-0.25) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.731 | **0.001** | 0.765 | 0.767 | 0.194 | 0.826 | 0.157 | 2.48 (+/-0.33) | 2.06 (+/-0.28) | 1.69 (+/-0.17) | 2.49 (+/-0.33) | 3.05 (+/-0.44) | 2.54 (+/-0.54) | 2.39 (+/-0.3) | 2.05 (+/-0.29) | 3.09 (+/-0.33) | 1.47 (+/-0.28) | 2.30 (+/-0.28) | 1.87 (+/-0.29) |
| *Gal* | galanin prepropeptide | 0.760 | **0.000** | 0.189 | 0.861 | 0.258 | 0.883 | 0.761 | 1.32 (+/-0.27) | 3.35 (+/-0.84) | 3.25 (+/-0.55) | 4.70 (+/-0.67) | 1.80 (+/-0.35) | 4.95 (+/-1.73) | 4.96 (+/-0.72) | 5.69 (+/-1.2) | 1.15 (+/-0.2) | 2.10 (+/-0.43) | 3.60 (+/-0.44) | 4.69 (+/-1.14) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.357 | 0.160 | 0.955 | 0.763 | 0.731 | 0.868 | 0.502 | 1.42 (+/-0.3) | 1.68 (+/-0.34) | 1.34 (+/-0.17) | 2.05 (+/-0.25) | 1.43 (+/-0.27) | 1.80 (+/-0.42) | 1.93 (+/-0.28) | 2.08 (+/-0.25) | 1.51 (+/-0.21) | 1.35 (+/-0.33) | 1.78 (+/-0.22) | 1.78 (+/-0.38) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.104 | 0.115 | 0.280 | 0.242 | 0.735 | 1.000 | 0.313 | 2.00 (+/-0.44) | 1.70 (+/-0.26) | 1.78 (+/-0.38) | 1.81 (+/-0.45) | 1.97 (+/-0.64) | 1.26 (+/-0.17) | 1.43 (+/-0.31) | 1.49 (+/-0.28) | 1.22 (+/-0.18) | 1.01 (+/-0.21) | 1.99 (+/-0.32) | 1.30 (+/-0.29) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.480 | 0.867 | 0.769 | 0.763 | 0.210 | 0.874 | 0.109 | 1.74 (+/-0.21) | 1.58 (+/-0.26) | 1.31 (+/-0.14) | 1.90 (+/-0.27) | 1.68 (+/-0.22) | 1.52 (+/-0.33) | 1.91 (+/-0.22) | 1.66 (+/-0.16) | 1.43 (+/-0.13) | 1.25 (+/-0.2) | 1.82 (+/-0.23) | 1.24 (+/-0.2) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.775 | **0.000** | 0.701 | 0.397 | 0.705 | 0.711 | 0.134 | 1.97 (+/-0.18) | 1.52 (+/-0.18) | 1.12 (+/-0.15) | 1.63 (+/-0.16) | 1.80 (+/-0.23) | 1.42 (+/-0.22) | 1.58 (+/-0.14) | 1.43 (+/-0.13) | 2.03 (+/-0.25) | 1.34 (+/-0.25) | 1.69 (+/-0.14) | 1.17 (+/-0.17) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.689 | **0.003** | 0.690 | 0.604 | 0.202 | 0.896 | 0.018 | 1.65 (+/-0.15) | 1.41 (+/-0.2) | 1.07 (+/-0.12) | 1.68 (+/-0.15) | 1.72 (+/-0.18) | 1.27 (+/-0.18) | 1.45 (+/-0.11) | 1.43 (+/-0.15) | 1.56 (+/-0.11) | 1.13 (+/-0.16) | 1.57 (+/-0.14) | 1.27 (+/-0.19) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.736 | **0.000** | 0.928 | 0.420 | 0.037 | 0.895 | 0.200 | 1.84 (+/-0.23) | 1.41 (+/-0.26) | 1.01 (+/-0.13) | 1.50 (+/-0.16) | 1.84 (+/-0.26) | 1.28 (+/-0.26) | 1.70 (+/-0.2) | 1.34 (+/-0.13) | 1.53 (+/-0.11) | 1.00 (+/-0.18) | 1.41 (+/-0.14) | 1.12 (+/-0.19) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.932 | 0.341 | 0.808 | 0.890 | 0.096 | 0.774 | 0.238 | 1.62 (+/-0.21) | 1.44 (+/-0.3) | 1.01 (+/-0.15) | 1.76 (+/-0.27) | 1.63 (+/-0.26) | 1.55 (+/-0.28) | 1.57 (+/-0.15) | 1.48 (+/-0.21) | 1.41 (+/-0.18) | 1.02 (+/-0.22) | 1.55 (+/-0.24) | 1.08 (+/-0.14) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.926 | **0.000** | 0.604 | 0.764 | 0.447 | 0.778 | 0.299 | 2.19 (+/-0.27) | 1.54 (+/-0.26) | 1.36 (+/-0.21) | 2.14 (+/-0.23) | 2.31 (+/-0.35) | 1.82 (+/-0.36) | 2.03 (+/-0.19) | 1.61 (+/-0.21) | 1.92 (+/-0.2) | 1.26 (+/-0.29) | 1.93 (+/-0.19) | 1.47 (+/-0.29) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.968 | **0.000** | 0.739 | 0.715 | 0.349 | 0.816 | 0.226 | 1.32 (+/-0.32) | 2.79 (+/-0.53) | 2.53 (+/-0.41) | 3.52 (+/-0.42) | 1.45 (+/-0.27) | 2.95 (+/-0.6) | 3.85 (+/-0.35) | 2.70 (+/-0.26) | 1.40 (+/-0.25) | 2.08 (+/-0.64) | 2.90 (+/-0.4) | 2.46 (+/-0.45) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.587 | **0.000** | 0.075 | 0.467 | 0.004 | 0.349 | 0.138 | 2.72 (+/-0.27) | 1.72 (+/-0.35) | 1.33 (+/-0.17) | 1.85 (+/-0.22) | 4.49 (+/-0.25) | 2.15 (+/-0.47) | 2.12 (+/-0.25) | 1.60 (+/-0.2) | 3.22 (+/-0.34) | 1.20 (+/-0.23) | 1.71 (+/-0.14) | 1.36 (+/-0.21) |
| *Igf1* | insulin-like growth factor 1 | 0.974 | 0.085 | 0.417 | 0.919 | 0.559 | 0.960 | 0.819 | 1.75 (+/-0.34) | 1.53 (+/-0.32) | 1.17 (+/-0.23) | 1.81 (+/-0.22) | 2.09 (+/-0.3) | 1.82 (+/-0.45) | 1.76 (+/-0.29) | 2.05 (+/-0.45) | 1.95 (+/-0.22) | 1.11 (+/-0.19) | 1.62 (+/-0.09) | 1.94 (+/-0.37) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.872 | **0.003** | 0.569 | 0.896 | 0.116 | 0.676 | 0.613 | 1.29 (+/-0.19) | 1.59 (+/-0.32) | 1.22 (+/-0.18) | 1.85 (+/-0.17) | 1.41 (+/-0.21) | 1.66 (+/-0.36) | 1.79 (+/-0.18) | 2.03 (+/-0.29) | 1.37 (+/-0.15) | 1.09 (+/-0.22) | 1.64 (+/-0.16) | 1.73 (+/-0.24) |
| *Kiss1* | KiSS-1 metastasis-suppressor | **0.000** | **0.000** | 0.337 | 0.276 | 0.592 | 0.971 | 0.729 | 1.77 (+/-0.34) | 2.15 (+/-0.63) | 5.75 (+/-0.97) | 5.13 (+/-0.64) | 2.02 (+/-0.29) | 2.18 (+/-0.76) | 9.88 (+/-2.16) | 6.14 (+/-1.39) | 1.63 (+/-0.34) | 1.75 (+/-0.49) | 6.85 (+/-1.83) | 4.89 (+/-1.02) |
| *Kiss1r* | KiSS1 receptor | 0.086 | **0.000** | 0.635 | 0.577 | 0.130 | 0.944 | 0.689 | 1.26 (+/-0.17) | 2.02 (+/-0.45) | 2.75 (+/-0.39) | 4.53 (+/-0.57) | 1.46 (+/-0.2) | 2.42 (+/-0.61) | 3.81 (+/-0.47) | 4.92 (+/-0.97) | 1.45 (+/-0.17) | 1.62 (+/-0.35) | 2.49 (+/-0.37) | 3.40 (+/-0.42) |
| *Lepr* | leptin receptor | 0.821 | **0.000** | 0.616 | 0.495 | 0.207 | 0.642 | 0.030 | 1.33 (+/-0.19) | 2.22 (+/-0.43) | 1.93 (+/-0.24) | 3.40 (+/-0.34) | 1.50 (+/-0.27) | 1.58 (+/-0.19) | 2.79 (+/-0.38) | 2.60 (+/-0.39) | 1.32 (+/-0.15) | 1.83 (+/-0.3) | 2.52 (+/-0.27) | 2.33 (+/-0.4) |
| *Mc3r* | melanocortin 3 receptor | 0.824 | 0.277 | 0.413 | 0.408 | 0.006 | 0.808 | 0.410 | 1.43 (+/-0.22) | 1.33 (+/-0.24) | 1.19 (+/-0.3) | 1.58 (+/-0.23) | 1.59 (+/-0.32) | 1.70 (+/-0.35) | 1.92 (+/-0.33) | 1.63 (+/-0.23) | 1.09 (+/-0.11) | 1.04 (+/-0.17) | 1.82 (+/-0.21) | 1.03 (+/-0.26) |
| *Oxt* | oxytocin, prepropeptide | 0.459 | **0.000** | 0.335 | 0.025 | 0.046 | 0.396 | 0.200 | 3.13 (+/-1.08) | 5.31 (+/-1.29) | 4.13 (+/-1.4) | 10.64 (+/-2.28) | 3.93 (+/-1.) | 6.74 (+/-2.01) | 12.22 (+/-3.76) | 21.56 (+/-5.96) | 4.14 (+/-0.81) | 2.20 (+/-0.53) | 4.28 (+/-0.56) | 14.66 (+/-4.14) |
| *Per2* | period homolog 2 | 0.949 | 0.016 | 0.710 | 0.389 | 0.070 | 0.896 | 0.569 | 1.96 (+/-0.43) | 1.63 (+/-0.33) | 1.54 (+/-0.21) | 2.22 (+/-0.28) | 1.95 (+/-0.33) | 1.85 (+/-0.39) | 2.77 (+/-0.43) | 2.59 (+/-0.43) | 2.27 (+/-0.41) | 1.14 (+/-0.23) | 1.93 (+/-0.22) | 1.97 (+/-0.42) |
| *Pgr* | progesterone receptor | **0.000** | **0.000** | 0.193 | 0.087 | 0.186 | 0.939 | 0.186 | 1.48 (+/-0.19) | 1.43 (+/-0.29) | 1.98 (+/-0.25) | 2.94 (+/-0.31) | 1.66 (+/-0.24) | 1.47 (+/-0.29) | 3.18 (+/-0.39) | 3.21 (+/-0.42) | 1.21 (+/-0.09) | 0.98 (+/-0.16) | 2.60 (+/-0.43) | 2.55 (+/-0.44) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.435 | 0.003 | 0.508 | 0.768 | 0.222 | 0.504 | 0.106 | 2.48 (+/-0.33) | 1.82 (+/-0.27) | 1.30 (+/-0.26) | 2.03 (+/-0.42) | 2.28 (+/-0.35) | 1.89 (+/-0.23) | 1.86 (+/-0.17) | 1.80 (+/-0.2) | 1.73 (+/-0.24) | 1.53 (+/-0.29) | 2.24 (+/-0.33) | 1.25 (+/-0.24) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.217 | 0.133 | 0.473 | 0.195 | 0.437 | 0.748 | 0.885 | 2.01 (+/-0.34) | 1.73 (+/-0.38) | 1.58 (+/-0.27) | 2.62 (+/-0.29) | 2.31 (+/-0.4) | 1.95 (+/-0.38) | 2.12 (+/-0.27) | 2.90 (+/-0.4) | 2.14 (+/-0.22) | 1.54 (+/-0.41) | 2.23 (+/-0.35) | 2.04 (+/-0.31) |
| *Tac2* | tachykinin 2 | **0.000** | **0.000** | 0.458 | 0.006 | 0.666 | 0.860 | 0.322 | 2.05 (+/-0.42) | 1.46 (+/-0.27) | 3.03 (+/-0.42) | 4.92 (+/-0.93) | 2.02 (+/-0.36) | 1.52 (+/-0.38) | 5.78 (+/-1.3) | 5.50 (+/-1.21) | 2.23 (+/-0.35) | 1.28 (+/-0.3) | 2.61 (+/-0.54) | 4.70 (+/-1.) |
| *Tgfa* | transforming growth factor, alpha | 0.224 | **0.000** | 0.602 | 0.449 | 0.490 | 0.431 | 0.578 | 1.85 (+/-0.35) | 1.48 (+/-0.31) | 1.36 (+/-0.19) | 2.47 (+/-0.32) | 1.89 (+/-0.27) | 1.47 (+/-0.31) | 2.24 (+/-0.33) | 2.45 (+/-0.37) | 1.99 (+/-0.2) | 1.05 (+/-0.18) | 1.89 (+/-0.24) | 2.00 (+/-0.38) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.594 | 0.004 | 0.639 | 0.828 | 0.101 | 0.549 | 0.927 | 1.80 (+/-0.35) | 1.62 (+/-0.34) | 1.38 (+/-0.25) | 2.16 (+/-0.18) | 1.92 (+/-0.33) | 1.61 (+/-0.34) | 2.04 (+/-0.2) | 2.43 (+/-0.36) | 1.92 (+/-0.27) | 1.09 (+/-0.25) | 1.85 (+/-0.26) | 1.85 (+/-0.35) |
| *Thra* | thyroid hormone receptor, alpha | 0.510 | 0.286 | 0.922 | 0.836 | 0.247 | 0.882 | 0.471 | 1.95 (+/-0.32) | 1.62 (+/-0.35) | 1.14 (+/-0.15) | 1.83 (+/-0.24) | 1.83 (+/-0.31) | 1.61 (+/-0.36) | 1.92 (+/-0.31) | 1.71 (+/-0.25) | 1.75 (+/-0.21) | 1.23 (+/-0.26) | 1.60 (+/-0.25) | 1.34 (+/-0.25) |
| *Thrb* | thyroid hormone receptor, beta | 0.966 | **0.000** | 0.889 | 0.406 | 0.090 | 0.919 | 0.428 | 1.39 (+/-0.23) | 2.86 (+/-0.64) | 2.61 (+/-0.3) | 3.90 (+/-0.56) | 1.56 (+/-0.26) | 2.61 (+/-0.55) | 3.83 (+/-0.3) | 4.14 (+/-0.6) | 1.19 (+/-0.12) | 1.97 (+/-0.36) | 3.26 (+/-0.51) | 3.28 (+/-0.58) |
| **Male Nonparametric Statistics** | | | | | | | | | | | | | | | | | | | | |
| Gene Symbol | Gene Name | Sex | Age | Trt | Sex/Age | Sex Trt | Age/Trt | Sex/Age /Trt | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 | P15 | P30 | P45 | P90 |
| *Avp* | arginine vasopressin | 0.050 | 0.023 | 0.447 | **0.000** | 0.267 | 0.049 | **0.000** | 19.81 (+/-8.35) | 5.59 (+/-1.33) | 2.85 (+/-0.87) | 5.64 (+/-1.77) | 8.78 (+/-2.9) | 2.30 (+/-0.5) | 3.97 (+/-0.83) | 6.03 (+/-1.81) | 32.00 (+/-10.) | 1.23 (+/-0.32) | 2.81 (+/-0.53) | 4.17 (+/-1.06) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.212 | **0.001** | 0.592 | **0.004** | 0.359 | 0.031 | 0.012 | 1.43 (+/-0.15) | 1.67 (+/-0.49) | 1.58 (+/-0.2) | 2.57 (+/-0.44) | 1.49 (+/-0.2) | 1.93 (+/-0.29) | 1.68 (+/-0.15) | 2.13 (+/-0.26) | 1.05 (+/-0.15) | 1.12 (+/-0.14) | 2.28 (+/-0.37) | 1.78 (+/-0.22) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.802 | **0.000** | 0.994 | **0.000** | 0.995 | **0.000** | **0.000** | 5.98 (+/-1.13) | 1.63 (+/-0.37) | 1.26 (+/-0.23) | 1.56 (+/-0.21) | 6.45 (+/-1.19) | 1.73 (+/-0.42) | 1.38 (+/-0.27) | 1.73 (+/-0.22) | 6.67 (+/-0.81) | 1.18 (+/-0.33) | 1.63 (+/-0.25) | 1.49 (+/-0.33) |
| *Gnrhr* | gonadotropin-releasing hormone receptor | 0.15 | 0.035 | 0.109 | **0.002** | 0.214 | 0.109 | 0.03 | 2.03 (+/-0.77) | 1.20 (+/-0.63) | 1.60 (+/-0.45) | 1.13 (+/-0.32) | 2.02 (+/-0.34) | 1.03 (+/-0.31) | 1.66 (+/-0.44) | 1.61 (+/-0.48) | 1.43 (+/-0.49) | 0.64 (+/-0.16) | 0.97 (+/-0.38) | 1.86 (+/-0.81) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.611 | 0.001 | 0.560 | 0.011 | 0.517 | 0.051 | 0.159 | 1.99 (+/-0.35) | 1.85 (+/-0.39) | 1.30 (+/-0.18) | 1.59 (+/-0.16) | 2.32 (+/-0.32) | 2.01 (+/-0.43) | 2.15 (+/-0.25) | 1.54 (+/-0.22) | 2.01 (+/-0.26) | 1.36 (+/-0.3) | 2.04 (+/-0.32) | 1.30 (+/-0.26) |
| *Mc4r* | melanocortin 4 receptor | 0.092 | 0.034 | 0.802 | 0.074 | 0.622 | 0.065 | 0.223 | 1.58 (+/-0.31) | 1.42 (+/-0.19) | 1.62 (+/-0.27) | 2.17 (+/-0.4) | 2.85 (+/-0.63) | 2.54 (+/-0.77) | 1.66 (+/-0.48) | 1.97 (+/-0.48) | 2.60 (+/-0.31) | 1.35 (+/-0.51) | 1.75 (+/-0.28) | 2.39 (+/-0.57) |
| *Oxtr* | oxytocin receptor | 0.086 | **0.000** | 0.558 | **0.000** | 0.282 | **0.005** | 0.009 | 1.89 (+/-0.32) | 1.86 (+/-0.4) | 1.48 (+/-0.25) | 3.17 (+/-0.68) | 2.06 (+/-0.33) | 1.55 (+/-0.34) | 2.94 (+/-0.61) | 2.93 (+/-0.59) | 1.82 (+/-0.23) | 1.10 (+/-0.19) | 2.40 (+/-0.32) | 1.75 (+/-0.55) |
| *Pdyn* | prodynorphin | 0.085 | 0.182 | 0.517 | 0.216 | 0.129 | 0.386 | 0.107 | 3.42 (+/-0.55) | 2.17 (+/-0.45) | 1.64 (+/-0.3) | 2.25 (+/-0.42) | 2.85 (+/-0.64) | 2.83 (+/-0.63) | 2.83 (+/-0.53) | 2.39 (+/-0.49) | 1.90 (+/-0.27) | 1.78 (+/-0.27) | 2.62 (+/-0.31) | 1.45 (+/-0.41) |
| **Assays Not Expressed or Did Not Amplify** | | | | | | | | | | | | | | | | | | | | |
| *Dnmt3l* | DNA (cytosine-5-)-methyltransferase 3l | | | | | | | | | | | | | | | | | | | |
| *Mc5r* | melanocortin5 receptor | | | | | | | | | | | | | | | | | | | |

EDC effects on gene expression in the ARC from P15 – P90 in males and females. Bold indicates effects that survived false discovery rate correction.

## DISCUSSION

Because of their long-term effects on reproductive function and physiology, endocrine disrupting chemicals have become a major focus of investigation seeking to identify molecular and epigenetic mechanisms underlying the increase in reproductive disorders observed in wildlife and human populations. EDCs are associated with reduced fertility and fecundity and increase risk of reproductive cancers. However, with the exception of a few studies (Cao et al., 2012; Ho et al., 2006; Prins et al., 2001) there is a dearth of studies examining the effects EDCs in a developmental context, especially in the hypothalamus. Here we sought to determine if exposure to an environmentally relevant dose of estrogenic EDCs alters the developmental profiles of neuroendocrine gene expression in the hypothalamus. Our lab and others have determined specific effects of A1221 on numerous reproductive parameters including disruption of estrous cycles (Chapter 3, (Dickerson et al., 2011b), altered reproductive behavior (Chung and Clemens, 1999; Steinberg et al., 2007), altered timing of puberty (Dickerson et al., 2011b) and reproductive senescence (Gellert, 1978b). Numerous studies have identified long-term effects of other EDCs on reproductive physiology and gene expression throughout the HPG axis (Reviewed in (Dickerson and Gore, 2007)). However, our study differs from the current literature in several fundamental ways. First we are the first to develop a comprehensive developmental profile of 48 neuroendocrine gene expression in 2 key hypothalamic nuclei necessary for reproductive function beginning in the juvenile period (P15) through puberty (P30 and P45) and continuing into adulthood (P90). We have also refined previous work (Dickerson et al., 2011b; Walker et al., 2012) by focusing in on

two of the key subnuclei, the AVPV and ARC, in the current analysis. Furthermore, we investigated effects in developing littermates to determine if gestational exposure to EDCs altered timing and progression of developmental processes dependent on hypothalamic function, allowing us to relate changes is reproductive physiology and function back to gene expression changes in the hypothalamus. Finally, we investigated if epigenetic mechanisms are disrupted by characterizing changes in DNA methylation of 2 of the genes that were significantly disrupted in a sex- and age-specific manner. Finally, investigating a large number of genes allowed us to identify novel gene targets and molecular mechanisms of endocrine disruption.

**Genes necessary for reproductive function are disrupted by exposure to EDCs in the AVPV but not the ARC**

One of the most surprising findings of this study is that *Kiss1* expression was not sexually dimorphic in the adult (P90) AVPV in our DMSO control animals. However, after exposure to EDCs in utero, there was a marked difference between the sexes. In addition, its expression was sexually dimorphic in the ARC of adult animals exposed to DMSO. It has been widely reported that *Kiss1* is sexually dimorphic in the adult AVPV but not the ARC (Reviewed in (Oakley et al., 2009)) therefore, it may be tempting to conclude that our vehicle control is masculinizing the hypothalamus. However, there is substantial evidence that this is not the case. First, the developmental profile of *Kiss1* observed in the AVPV and ARC of our animals exposed to DMSO replicates previous work in untreated animals from our lab (POA and MBH) (Walker et al., 2012) and closely resembles that of others, including evidence for sex differences in the ARC

(females on diestrus; AVPV and ARC specific) (Cao and Patisaul, 2011; Takumi et al., 2011). Second, the expression patterns of *Tac2* in our DMSO controls replicate those published by Navarro et al. (Gill et al., 2012) (ARC of females) and our lab (MBH dissections; (Walker et al., 2012) in untreated animals as do the sex differences in expression of *Tac2* in the ARC (Ruiz-Pino et al., 2012; Walker et al., 2012). Finally, *Esr1* (Cao and Patisaul, 2011; DonCarlos and Handa, 1994; Lauber et al., 1991a; Zhou et al., 1995) and *Pdyn* (Simerly et al., 1996) have been reported to be sexually dimorphic in the AVPV and organized by sex steroid hormones (*Esr1*: (Cao et al., 2012; Dickerson et al., 2011b; Simerly and Young, 1991); *Pdyn*: (Simerly, 1991)). However, expression is highly dependent on cycle stage of the females, as differences are most pronounced when the females are in diestrus (*Esr1* (Zhou et al., 1995) and *Pdyn* (Simerly et al., 1996)) or estrus (*Pdyn* only) but not on proestrus when our comparisons were made.

The 4 genes highlighted in Figure 2.1 have been identified as key players in regulating the synthesis and release of GnRH and initiating the timing of puberty. Additionally, in the ARC, they are all expressed in the same neurons, commonly referred to as the KNDy neurons (Navarro et al., 2009). While it is generally recognized that the kisspeptin neurons in the AVPV are integral for generating the preovulatory GnRH/LH surge (Smith et al., 2006) the functional role of the KNDy neurons in the ARC has not been fully characterized. In rodents, it has been proposed that these neurons regulate negative feedback of GnRH through the actions of circulating sex steroid hormones (Kauffman et al., 2009) and that these neurons, especially *Tac2*, serve as the pacemaking signal for pulsatile GnRH release (Navarro, 2012). Recent evidence in ewes suggests that

they also play a role in the preovulatory surge (Merkley et al., 2012; Smith et al., 2009). Data from our current study support the pacemaking hypothesis and suggest that the KNDy neurons may play a role in the preovulatory surge, potentially by regulating the increase in pulse amplitude and frequency of LH during the surge.

Previous reports from our lab indicate that the LH surge may be disrupted in females exposed to A1221 (Steinberg et al., 2008). Therefore, our goal was to euthanize our females during their LH surge (1 -3 hours before lights out on proestrus) (Scarbrough and Wise, 1990; Urbanski and Ojeda, 1986). Not only does this provide us with information regarding how the surge may be affected by EDCs but also provides insight into mechanisms of the surge. For example, in the ARC of our animals, the levels of the neuropeptides known to stimulate GnRH (kisspeptin and neurokinin B) are significantly greater in the adult female than the male. These data suggest that these neurons may play a role in the LH surge by sending more excitatory signals to GnRH neurons in females during this time. The alterations in estrus cyclicity observed in our treated females support this hypothesis. Females exposed to EB had significantly fewer animals with regular cycles. For A1221, effects on the cycle were more subtle; however, both EDC treatment groups tended to have at least 2 elongated series (5 or more days) of cornified and/or leukocytic smears ($p < 0.1$) and were more likely to transition from diestrus to estrus (thus skipping the proestrus phase) (EB $p = 0.003$; A1221 $p = 0.16$) when compared to the DMSO controls. Additionally, both EDC-treated groups of females had increased expression of *Kiss1*, *Tac2*, *Esr1* and *Pdyn* on P90 in the AVPV when compared to their control counterparts, while no effects of treatment were observed in the ARC.

Therefore, we hypothesize that the differences in cycles are the result of dysregulation of the excitatory and inhibitory signals converging on the GnRH system from the ARC and AVPV.

**Disruption of gene expression in the AVPV may be the result of altered circadian function**

While there were a few effects of treatment on gene expression in the male AVPV (Figure 2.3A), most (21) of the 28 genes affected by treatment were specifically affected in the female AVPV. Interestingly, we noticed that many of the expression profiles appeared to resemble those observed in the males, which is difficult to test using traditional statistics. Therefore, we utilized a combination of analytical methods to determine if genes expression profiles in the EDC females' AVPVs were more similar to that of control females than males (i.e., were the EDC females more similar to DMSO females, or were they more similar to DMSO males, the latter suggesting masculinization). Using the criteria described above, we determined that 9 genes in the female AVPV exposed to EB or A1221 closely resemble the DMSO male (Figure 2.2) but not the DMSO female suggesting that these 9 genes may be "masculinized" by treatment. Many of these genes were decreased on P15 and increased on P90 when compared to the DMSO females. However, during the pubertal transition (P30 – P45) few effects were observed in the A1221 treated females, while in the EB treated females, expression was increased on P45 when compared to all other groups (*Gnrh1*, *Kiss1r*, *Gal* and *Per2*). There is evidence that the structural changes in the female AVPV aren't initiated until ~P30 and the nucleus does not become fully sexually dimorphic until P60 –

P90 even though it is organized by neonatal hormone exposure (Davis et al., 1996b). We hypothesize that the age and sex specific differences in gene expression observed between the control females and the DMSO males /treated females reflect this process, with the P15 changes in gene expression reflecting the initiation and the P90 changes reflecting the completion of sexual differentiation of the AVPV.

One novel finding of our study was that the 2 clock genes (*Arntl* and *Per2*) included on our array were different between the sexes in the AVPV of our DMSO animals on P15 (trend; *Arntl* p =0.06; *Per2* p = 0.119) and P90 (*Arntl* p = 0.003; *Per2* p = 0.018) and furthermore, that gestational exposure to EDCs resulted in a male typical expression profile throughout development in the female AVPV. Other studies have investigated the sex differences in clock genes in the SCN (Iwahana et al., 2008; Karatsoreos et al., 2011) and other tissues (Hirao et al., 2011; Textoris et al., 2010) as well as how EDCs (notably dioxin) affect clock gene expression and function throughout the body (Mukai et al., 2008; Mukai and Tischkau, 2007; Tischkau et al., 2011). To our knowledge we are the first to show that clock genes in the AVPV are sexually dimorphic, a target of EDCs, and that expression can be programmed by gestational exposure to estrogenic compounds. A recent study focusing on clock genes in the suprachiasmatic nucleus (SCN), the central regulator of circadian rhythms, reported that rhythmic expression of *Arntl* and *Per2*, among other clock genes, was activated between E19 and P3 with the rhythms becoming more pronounced by P10 in juvenile rats (Sladek et al., 2004). While sex differences were not investigated, these data provide evidence that the

rhythmic expression clock genes is developing during the critical period of sexual differentiation of the brain.

Recent evidence suggests that the AVPV plays an important role in circadian regulation of GnRH synthesis and release. Our current study supports and expands on this hypothesis. While it has not been investigated in males, *Kiss1* displays rhythmic expression in the female AVPV (Robertson et al., 2009; Smarr et al., 2012; Xu et al., 2011) and is directly regulated by the circadian transcription factor DBP. We hypothesize that the lack of sexually dimorphic expression of *Kiss1* in our untreated (Walker et al., 2012) and DMSO animals is a reflection of circadian expression of *Kiss1* in the *male* AVPV and is a plausible explanation as to why *Kiss1* expression is sexually dimorphic in the AVPV of treated animals but not the DMSO animals. There is a paucity of data investigating the function of the male AVPV. To our knowledge this is the first study to provide evidence that the AVPV maybe important for regulating circadian GnRH release in both males and females. While further research is necessary, there is evidence that there are sex differences in diurnal expression of *Gnrh* mRNA in the POA (Gore, 1998; Gore and Roberts, 1995; Gore et al., 1996) as well as sex differences in diurnal LH release (Gore, 1998; Urbanski and Ojeda, 1985). Finally, *Per2* protein and mRNA expression in the SCN is modulated sex steroid hormone fluctuations in females during the estrous cycle (Perrin et al., 2006) and in males after gonadectomy (Karatsoreos et al., 2011). We propose that Kiss1 neurons in the male AVPV regulate the circadian release of LH, whereas, in the female it is necessary to integrate hormonal and circadian cues in order to regulate the preovulatory LH surge. These functional differences between the

sexes are organized during the perinatal period, develop through the juvenile period, and culminate in adulthood as sexually dimorphic release of GnRH.

**Subtle differences in DNA methylation may be programmed by gestational exposure to EDC**

We measured DNA methylation of promoter regions of 2 genes (*Per2* and *Ar*) displaying sex differences in the AVPV and displaying male typical expression patterns in the treated females. The *Per2* promoter region was almost completely demethylated by P15, an effect that has been reported in the SCN (Ji et al., 2010). There were a few significant effects of treatment (data not shown), however, because the level of methylation was so low (< 5% at most sites) the tissue used to obtain the DNA sample was heterogeneous these results may not be biologically relevant or reproducible. As expected, methylation of the promoter region of *Ar* was sexually dimorphic. Because *Ar* is on the X-chromosome, methylation should be ~50% in the female due to X-inactivation. A significant treatment effect was observed for one CpG in the male AVPV, but this should be interpreted with caution as this site was almost completely demethylated and the change was less than 5%. By investigating a gene on the X-chromosome we are able to conclude that EDCs are probably not altering global methylation. If this were the case, we would expect that these CpG would be escaping X-inactivation, an effect that was not observed. Finally, we noted small but significant differences in correlations of specific CpGs with Ar expression. More specifically, in the treated females *Ar* expression was positively correlated with methylation of several CpGs investigated, an effect that was observed in the DMSO males but not the DMSO females.

Interestingly, two of these sites (-57 and -51) are a putative binding site for REV-ERBα, a transcriptional repressor of circadian gene expression (Guillaumond et al., 2005; Preitner et al., 2002) providing support for our hypothesis that the AVPV is an important regulator of circadian signaling. Again, this needs further testing in future experiments.

**Gene expression changes in the ARC are specific to males and may provide information regarding the timing of puberty**

As mentioned, the ARC regulates negative feedback onto the GnRH system and is not considered to be a sexually dimorphic nucleus in the hypothalamus in terms of morphology (Reviewed in (Navarro, 2012)). Therefore, we were not expecting gestational exposure to EDCs to results in sex specific effects in the ARC. Contrary to this prediction, 12 of the 48 genes measured were altered by gestational exposure to EDCs (main effect or interaction of treatment $p < 0.05$). Of those, 6 were affected specifically in the male ARC. In addition, males exposed to A1221 went through puberty later than their DMSO counterparts. Therefore, we hypothesize that genes altered in the male ARC by A1221 may be important in regulating the timing of puberty. Consistent with this idea, there is some limited evidence that the ARC is important for regulating the timing of reproductive transitions (Navarro et al., 2011b). With specific reference to A1221, two genes, *Ar* and *Lepr*, were significantly altered. These genes are critical for the hypothalamus to sense and respond to peripheral changes in metabolism (*Lepr*) and gonadal status (*Ar*) – two of the permissive cues that are known to drive the onset of puberty (Ebling, 2005). The expression profiles of both genes displayed similar

expression profiles but were "out of phase" with the DMSO males – when expression was up in the DMSO animals it was suppressed in the A1221 animals and vice versa.

## SUMMARY AND CONCLUSIONS

Numerous studies have focused on gene expression in the adult hypothalamus after gestational exposure to EDCs. However, many have focused on one developmental time point or sex. (Reviewed in (Dickerson and Gore, 2007)). Here we present a comprehensive developmental profile of gene expression in two nuclei of the hypothalamus and relate them back to altered reproductive physiology and development. This approach has enabled us to identify novel targets of EDCs, notably the clock genes *Per2* and *Arntl*, as well as develop new hypotheses regarding the functional outcomes of sexual differentiation of the brain. Finally, characterizing gene expression changes in a developmental context provide valuable insight into how we define a masculinized phenotype. Many of our genes did not show differences during the pubertal transition but were similar to the male throughout development. It is important to consider developmental and cycle stage when investigating the effects of gestational exposure to EDCs.

# CHAPTER 3

## Disruption of Reproductive Aging in Rats by Gestational Exposure to Estrogenic Endocrine Disruptors


### ABSTRACT

Polychlorinated Biphenyls (PCBs) are industrial contaminants and known endocrine-disrupting chemicals (EDC). Previous work has shown that gestational exposure to PCBs cause changes in reproductive neuroendocrine processes. Here, we extended work farther down the life spectrum, and tested the hypothesis that early life exposure to Aroclor 1221 (A1221), a mixture of estrogenic PCBs, results in sexually dimorphic aging-associated alterations to reproductive parameters in rats, and gene expression changes in hypothalamic nuclei that regulate reproductive function. Pregnant Sprague-Dawley rats were injected on gestational days 16 and 18 with vehicle (DMSO), A1221 (1mg/kg) or estrogenic control [estradiol benzoate (EB, 50 $\mu$g/kg)]. Developmental parameters, estrous cyclicity (females), and timing of reproductive senescence were monitored in the offspring through 9 months of age. Expression of 48 genes was measured in three hypothalamic nuclei, the anteroventral periventricular nucleus (AVPV), arcuate nucleus (ARC), and median eminence (ME; female only) by real-time RT-PCR. Serum LH, testosterone, and estradiol (E2) were assayed in the same animals. In males, A1221 had no effects; however, prenatal EB increased serum E2, gene expression in the AVPV (1 gene) and the ARC (2 genes) compared to controls. In females, estrous cycles were longer in the A1221 exposed females throughout the lifecycle. Gene expression was not affected in the AVPV but significant changes were

caused by A1221 in the ARC and ME as a function of cycling status. Bionetwork analysis demonstrated fundamental differences in physiology and gene expression between cycling and acyclic females independent of treatment. Thus, gestational exposure to biologically-relevant levels of estrogenic EDCs has sexually dimorphic effects, with an altered transition to reproductive aging in female rats, but relatively little effect in males.

## INTRODUCTION

In mammals, reproductive aging is associated with the loss of reproductive capacity and involves dysregulation of the hypothalamic-pituitary-gonadal (HPG)-axis. In the hypothalamus, there are transcriptional (Gore et al., 1996), translational (Chakraborty et al., 2003a; Chakraborty et al., 2003b; Lederman et al., 2010; Sahu and Kalra, 1998; Zuo et al., 1996) and ultrastructural morphological changes (Yin et al., 2009a) culminating in altered expression and release of gonadotropin-releasing hormone (GnRH), the obligatory decapeptide for reproductive function. Additionally, feedback of gonadal sex steroid hormones on the hypothalamus and the pituitary gland becomes eroded during the process of reproductive aging. While all levels of the HPG axis undergo age-related changes, there is mounting evidence suggesting that changes in the neuronal/glial network regulating GnRH release from the hypothalamus plays a key role in the process of reproductive senescence, particularly in the rodent model (Kermath and Gore, 2012).

Recently, it has become clear that the process of reproductive aging is regulated by both genetic and environmental factors. Genetic studies reveal numerous genes

associated with the menopausal transition, especially in humans (Reviewed in (Kok et al., 2005)). Other studies have focused on the role of the environment and especially endocrine disrupting chemicals (EDCs) in the reduction of fertility and fecundity in both sexes (Reviewed in (Dickerson and Gore, 2007)). EDCs are "an exogenous chemical, or mixture of chemicals, that interfere with any aspect of hormone action" (Zoeller et al., 2012) and include plastics and plasticizers (phthalates, BPA), pharmaceuticals (DES), pesticides (DDT) and industrial contaminants (PCBs, dioxins), among others. Because hormone systems regulate physiological processes such as metabolism, reproduction, and stress, these compounds undoubtedly have adverse effects on endocrine health in wildlife and humans. Exposure to EDCs during critical periods of development (e.g. the perinatal period), when cellular and molecular changes are most profound, can result in lifelong alterations and an increased disease burden in adulthood – a concept referred to as the fetal basis of adult disease (Reviewed in (Barker, 2003)). With regard to reproductive senescence, emerging evidence suggests that exposure to EDCs during a critical period of development hastens the transition to acyclicity and causes other reproductive changes in female rodents (Adewale et al., 2009; Armenti et al., 2008; Dickerson and Gore, 2007; Gellert, 1978a, b; Gore et al., 2011; Shi et al., 2007). However, to our knowledge, no study has investigated the effects of gestational exposure to EDCs on the aging male hypothalamus.

Here, we investigated sexually dimorphic effects of perinatal exposure to a lightly chlorinated mixture of PCBs, Aroclor 1221 (A1221) on the aging hypothalamus of rats. PCBs are industrial contaminants used in the US from the 1930s - 70s. Although these

compounds have been banned for decades, exposure continues through contamination of soil, water and food sources as well as bioaccumulation. In this study, we examined whether gestational exposure to A1221 resulted in 1) altered timing and progression of reproductive senescence (acyclicity) in female rats; 2) alterations in gene expression in key brain regions associated with the progression of reproductive aging in females; and 3) changes in male reproductive physiology and gene expression throughout the lifecycle. Our overarching goal was to determine the molecular underpinnings of hypothalamic reproductive aging and how those processes might be disrupted by perinatal exposure to EDCs.

## MATERIALS AND METHODS

### Animals

All animal protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by The University of Texas at Austin Institutional Animal Care and Use Committee. Sprague-Dawley rats (3-4 month virgin females) were purchased from Harlan Laboratories (Houston, TX) and impregnated in house in a manner similar to work previously conducted in our laboratory (Dickerson et al., 2011a; Dickerson et al., 2011b). Because standard soy-based rat chow contains phytoestrogens, animals were switched to low phytoestrogen Harlan-Teklad 2019 Global Diet ad libitum. Animals were housed individually under constant humidity and temperature (21-22°C) with a partially reversed 12:12 light cycle (lights on at 23:00). Rats were given at least 1 week to adjust to the new diet and environment before mating and were on the new diet for 2-3 weeks before conception. The morning after successful

mating (sperm positive vaginal smear) was termed embryonic day (E) 1. On E16 and E18, during the period of sexual differentiation of the rat brain (Davis et al., 1996b; Jacobson et al., 1980; Rhees et al., 1990a, b), dams were weighed and randomly assigned to one of 3 treatment groups and injected with 0.1ml vehicle (DMSO 99.5% Sigma #D4540); 50 $\mu$g/kg estradiol benzoate as a positive estrogenic control (EB, Sigma #E8515); or 1 mg/kg Aroclor 1221 (A1221; AccuStandard, #C221N). Doses and routes of exposure were chosen based on previous work in our laboratory (Dickerson et al., 2011a; Dickerson et al., 2011b; Steinberg et al., 2007; Steinberg et al., 2008) and others (Chung and Clemens, 1999; Gillette et al., 1987a; Gillette et al., 1987b; Murugesan et al., 2005a; Murugesan et al., 2005b). Although we did not save carcasses to measure body burden, we estimate that each pup is exposed to ~1:500 of the dose (i.e., 2 $\mu$g/kg A1221 or 100 ng of EB) based on previous literature (Takagi et al., 1986). Studies investigating human exposure have found that concentrations in maternal serum (Karmaus and Zhu, 2004; Law et al., 2005; Longnecker et al., 2005; Schantz, 1996), cord blood (Lackmann, 2002) and milk fat (Schantz, 1996) are between 1 and 9 ppb. Therefore, our dose is estimated to be within the range of human exposure. Because of differences in metabolism of the estrogenic control (EB) (Eaton et al., 1975; Landau, 1976) and A1221 (Anderson et al., 1977; Heinrich-Hirsch et al., 1997; Lutz et al., 1984; Mathews and Anderson, 1975; Matthews and Dedrick, 1984; Tampal et al., 2002), and to maintain consistency with other experiments in our laboratory (Dickerson et al., 2011a; Dickerson et al., 2011b; Steinberg et al., 2007; Steinberg et al., 2008), injections were given via one of two different routes depending on treatment: EB and half of the DMSO treatments

were given as subcutaneous injections, and A1221 and half of the DMSO treatments were given as intraperitoneal injections. Any effects of route were considered as a covariate for analysis. On the day after birth [postnatal day (P) 1], litter composition, birth weights and anogenital distance were recorded, and the litters were culled to equal sex ratios. Body weights were monitored weekly throughout the lifecycle and anogenital distance was measured weekly until weaning. Pups were weaned on P21 and housed with same sex littermates (2-3 per cage) until P90. For a companion study, littermates were euthanized on P15, 30, 45 and 90. Rats were monitored for a secondary sex characteristic of the onset of puberty daily (preputial separation in males and vaginal opening in females). Beginning on P90, animals were randomly assigned a new cage mate based on the age of each litter. Investigators were blind to treatment throughout the experiment. Therefore, cage mate treatment was not taken into account when rehousing rats. Because of the large number of animals necessary for both studies, animals were raised in 5 cohorts with treatments equally distributed across each cohort.

**Tissue Collection and Storage**

Females were classified as reproductively cyclic (regular or irregular cycles) or as acyclic (persistent estrus; see below for more details). Those females in persistent estrus were euthanized after at least 14 consecutive days of cornified smears and as close to 9 months of age as possible. Unless females were in persistent estrus, they were euthanized on the day of proestrus. Final sample sizes for females were Cyclic DMSO: 13; Acyclic DMSO: 8; Cyclic EB: 11; Acyclic EB: 6; Cyclic A1221: 9; Acyclic A1221: 13. For males, 1 male/ litter (DMSO = 22; EB =18 and A1221 = 22) was included in the analysis

of the somatic and hormonal data, however only a subset of hypothalamic punches were used to measure gene expression (DMSO: 11, EB: 8, A1221: 11). Rats were euthanized by rapid decapitation approximately 1 to 3 hours before lights out. Brains were removed and sectioned in 1 mm coronal sections using an ice-cold stainless steal brain matrix. Sections were placed on an ice-cold microscope slide and snap frozen on dry ice. Frozen sections were placed on a freezing stage, allowed to equilibrate to -15 to -20°C, and micropunches (0.98 mm diameter) were taken from each region of interest and placed in a frozen eppendorf tube. Photographs were captured of sections before and after punches were taken to ensure consistency across the cohorts. Trunk bloods were collected, allowed to clot, and serum was separated via centrifugation (1500 X g for 5 min). Tissues and serum were stored at -80°C until use.

**RNA Extraction**

RNA was extracted from frozen anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of males and females using a Qiagen Allprep DNA/RNA mini kit and from frozen median eminence (ME – female only) dissections using a Qiagen Allprep DNA/RNA micro kit due to the smaller tissue volume, all according to manufacturer's protocols. RNA samples were eluted with RNase free water and treated with 1 U of TURBO® DNase (Applied Biosystems Inc., Foster City, CA) to rid samples of genomic DNA before ethanol precipitation. Resuspended samples were diluted to a concentration of 50 ng/$\mu$l (AVPV, ARC) or 1 ng/$\mu$l (ME) based on nanodrop and bioanalyzer analysis. All samples were run on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) to assess RNA concentration, purity and integrity.

**Taqman® Microfluidic Real-time PCR Cards**

Samples were run on custom-designed microfluidic 48-gene PCR cards (Applied Biosystems Inc, Foster City, CA), with specific gene assays chosen based on a priori hypotheses and published reports on their importance in neuroendocrine function and sensitivity to disruption by EDCs (46 genes of interest and 2 housekeeping genes; Tables 3.1, 3.2, 3.4, 3.5). For technical reasons ME samples were run on a separate TLDA array (Table 3.3), which was composed of a slightly modified panel of neuroendocrine genes. We did not have enough arrays from this lot to run the male MEs, which could not be assayed in this study. Messenger RNA (200 ng AVPV, ARC; 10 ng ME) was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA) according to manufacturer's protocol. Product was stored at -20°C until use.

Real-time PCR was carried out on an ABI ViiA7 using Taqman® Universal Mastermix (Applied Biosystems, Foster City CA) and the following run parameters: 50°C for 2 minutes, 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression was determined for each sample using the comparative Ct Method (Livak and Schmittgen, 2001; Pfaffl, 2001; Schmittgen and Livak, 2008). Samples were normalized to either 18s or Gapdh and calibrated to the median delta-Ct of the DMSO controls to determine relative change in expression for each gene.

**Serum hormone assays**

Serum LH was measured in duplicate 50 $\mu$l samples in the laboratory of Dr. Michael Woller, University of Wisconsin-Whitewater, by double antibody competitive

binding radioimmunoassay (RIA). The assay was performed using the rat LH RP-3 standard from the National Hormone and Pituitary Program of the NIDDK (kindly provided by Dr. A.F. Parlow). All samples were measured in a single assay, and the intra-assay variability was 3.85%.

Serum concentrations of sex steroid hormones were measured using RIA for estradiol (Beckman Coulter, Webster, TX) and testosterone (MP Biomedicals) according to the manufacturer's recommended protocols. Samples were run in duplicate if possible. Samples having a CV of 10% or greater were rerun when possible or dropped from analysis.

Two estradiol assays, were run with assay sensitivity of 2.2 pg/ml and intra-assay variability of 6.48% and 1.64% respectively. Inter-assay CV was 4.55%. One testosterone assay was run with assay sensitivity of 0.03 ng/ml and intra-assay variability of 1.40%.

**Statistics**

Data in females were analyzed for interactions of reproductive cycle status and treatment for all somatic markers recorded as well as gene expression data. Females were classified as either cyclic or acyclic using the smear data from the last 30 days of life. We categorized a female as cyclic if she had greater than 4 nucleated (indicative of proestrus) days in the last 30 days (regular cyclers should have at least 5), or as acyclic if there were fewer than 4 nucleated days during the last 30 days of life and/or the female was in persistent estrus for at least 14 days. For gene expression data, statistics were performed using relative expression values for each sample. For females, multiple regression analysis was conducted using PASW® software (IBM, Armonk NY) to compare each

endpoint (genes, hormones, and endocrine tissues) using cycle status and treatment as independent variables. For those endpoints where a significant main effect of cycle status or treatment or an interaction was observed data were split by independent variables and follow-up analysis was performed to determine specific differences between each group. For the males, a one-way ANOVA was conducted to compare each endpoint using treatment as an independent variable. In both sexes, if data did not meet the assumptions for multiple regression analysis, data were transformed (natural log or square root) and reanalyzed. In a few cases, transformed data did not meet assumptions for statistical analysis by multiple regression. In those cases, data were analyzed using a Kruskal-Wallis test followed by a Mann-Whitney test between each group. For hormone concentrations, an effect was considered significant at $p<0.05$. For gene expression data a Benjamini and Hochberg False-Discovery Rate correction (Benjamini and Hochberg, 1995; Hochberg and Benjamini, 1990) was used to correct our p-values to account for the large number of variables measured. Parametric data were tested for outliers using the z-score of the residuals from the initial regression. A data point was considered an outlier if the residual was greater than 2.5 standard deviations from initial line of best fit. Non-parametric data were tested for outliers using the Grubbs' outlier test. Confirmed outliers were excluded from final analysis.

Vaginal smear data were analyzed with a python script using rule-based pattern matching techniques to determine the number and length of each cycle for each animal. Briefly, cycles were identified for each animal by scanning the smear data from the day of vaginal opening until euthanasia for the diestrus 2 to proestrus transition. The make-up

and length of each cycle was recorded throughout the lifecycle. A repeated-measures ANOVA was conducted using the moving average of the cycle length (using three cycle lengths) for each animal to account for any missing data.

A bootstrap technique (Efron and Tibshirani, 1993) that has been previously employed by our laboratory (Walker et al., 2012) was used to examine possible relationships between gene expression, endocrine tissues and serum hormones throughout development using Matlab (The Mathworks, Natick MA) software. Briefly, original paired data were resampled with replacement for 1000 repetitions, and a Pearson's correlation coefficient was computed on each of these new bootstrapped data sets to build a distribution of coefficients. Significance of Pearson's correlation coefficient for each interaction was determined from the bootstrapped distributions. Only those correlation coefficients that survived a Benjamini and Hochberg False Discovery Rate (Benjamini and Hochberg, 1995; Hochberg and Benjamini, 1990) at a p-value $< 0.05$ were considered significant.

Hierarchical cluster analysis and heatmaps were performed using Multiple Experiment Viewer V4.8.1 (TM4.org) and clusters were validated using R statistical packages.

## RESULTS

Animals exposed to A1221 during gestation were monitored daily from birth to 9 months of age to determine if perinatal exposure to environmental endocrine disruptors altered reproductive aging in males and females. We measured changes in reproductive tissues, tracked female estrous cycles, and measured 48 genes using a Taqman low-density array in 3 brain regions selected for their importance in reproductive function:

AVPV and ARC (male and female) and ME (female only). While no genes survived a Benjamini and Hochberg False Discovery Rate correction (Benjamini and Hochberg, 1995; Hochberg and Benjamini, 1990), all genes had been specifically chosen based on our a priori hypotheses about their known neuroendocrine functions, therefore we have reported gene expression data as significant ($p<0.05$) or as a trend ($p<0.1$).

**Effects of prenatal EDCs on females – somatic measurements, hormones, and estrous cycles**

In females, few effects of treatment were observed on pituitary weight, gonadal somatic index, or uterine weight (Figure 3.1). However, there were main effects of cycle status between cyclic and acyclic females. In the acyclic animals, pituitary weights were increased, and gonadal somatic index (GSI) and uterine weights were decreased compared to cyclic counterparts. Serum LH concentrations in cyclic control (DMSO) rats were substantially higher than in cyclic EB or A1221 rats. The lack of significance in this dataset is likely attributable to the pulsatile nature of LH release. For serum estradiol, there were no significant effects of cycle status or treatment.

Repeated-measures ANOVA analysis of the moving average of the cycle length indicated there was a significant effect of treatment ($p=0.03$) on cycle length throughout the lifecycle (Figure 3.2). Post-hoc analysis revealed that females exposed to A1221, but not EB, had significantly longer cycles throughout their lifecycle when compared to the DMSO females, and that the length of cycles increased with age ($p<0.001$) in all treatment groups.

**Effects of prenatal EDCs on females – gene expression in AVPV, ARC, and ME**

Analysis of gene expression in hypothalamic regions of females revealed that cycle status (cyclic or acyclic) played a substantial role in results. Therefore, data are presented for effects of prenatal treatment, cycle status, and interactions of treatment by cycle status. Significant effects ($p < 0.05$) and a trends ($0.05 < p < 0.075$) are graphed. Data for all the genes detected in each hypothalamic subregion are provided in Supplemental Tables. In the female AVPV (Table 3.1), only one gene was significantly altered by treatment: *Grin2c* ($p=0.046$) was increased in EB treated females compared to DMSO controls.

In the ARC (Figure 3.3, Table 3.2), 2 genes were significantly affected by treatment: *Lepr* and *Gnrhr*. One gene, *Oxt,* was significantly altered by cycle status. A total of 14 genes displayed a significant status by treatment interaction ($p<0.05$; Table 3.2) and are also indicated in red on Figure 3.5B. Further analysis revealed that 8 of these latter mRNAs (*Dnmt3a*, *Ar*, *Pdyn*, *Lepr*, *Mc3r*, *Oxt, Grin2b, Grin2d*; Figure 3.3) had lower expression in the acyclic A1221 females when compared to acyclic DMSO counterparts. One gene, Esr1, was higher in the cyclic A1221 females when compared to cyclic DMSO and cyclic EB counterparts (Figure 3.3). As a whole, affected genes fell into four functional categories: epigenetic processes (*Dnmt3a*); steroid hormone receptors (*Esr1* and *Ar*); neuropeptide signaling (*Pdyn*, *Lepr*, *Mc3r* and *Oxt*); and glutamate signaling (*Grin2b* and *Grin2d*).

In the ME (Table 3.3), there were no significant main effects of treatment or reproductive status. However, four genes displayed significant status by treatment

interactions (Figure 3.4; also see red text on Figure 3.5C) in two functional categories: 1) steroid hormone receptors (*Esr1, Nr3c1*) and 2) neurotransmitter systems (*Grin2b, Gad2*). As shown in Figure 3.4, these four genes were expressed at higher levels in DMSO cycling than DMSO acyclic rats (significant for *Nr3c1* and *Grin2b*; trend for *Esr1*). By contrast, this cycling status effect was not seen in EB or A1221 rats. In addition, DMSO cyclic females had higher *Esr1* mRNA levels compared to EB and A1221 cyclic rats, and a trend for higher *Gad2* mRNA compared to EB cyclic rats.

**Effects of prenatal EDCs on males – estradiol and gene expression in AVPV and ARC**

In males, treatment had no effect on body weight, pituitary weight, gonad weight, serum LH, or serum testosterone (Table 3.6). A main effect of treatment was observed on serum estradiol in males ($p=0.022$), although post-hoc analysis revealed only trends for higher estradiol levels in the EB ($p=0.066$) and A1221 ($p=0.053$) males when compared to their DMSO counterparts. One gene was altered in the male AVPV: *Avpr1a* ($p = 0.05$; Table 3.1). Two genes were significantly altered in ARC of males: *Bdnf* ($p=0.008$) and *Ahr* ($p=0.006$), with trends for *Slc17a6* and *Oxt*. The complete male datasets are shown in Tables 3.4 (AVPV) and 3.5 (ARC).

**Hierarchical cluster analysis of relationships by cycle status and treatment in females**

In order to determine if specific gene expression patterns within each brain region are related by treatment and cycle status in females, hierarchical cluster analysis was conducted and clustergrams were generated for each brain region (Walker et al., 2012). Male cluster analysis was not performed because there were too few groups to be able to

validate the clusters. Multiple experiment viewer (MeV 4.8.1) software was used generate the heat maps and to perform the analysis using the average linkage method and the correlation coefficients to express similarity. Clusters were validated using R statistical packages.

Clusters by cycle status and treatment revealed only treatment differences in the AVPV (Figure 3.5A, top) with EB females clustering separately from DMSO and A1221 ($p=0.08$). However, no validated gene clusters (Figure 3.5A, side) were observed in the AVPV so it is difficult to determine which genes might be driving the differences observed in the clustering of the EB animals.

In the ARC, DMSO cyclic females tended to cluster with the acyclic A1221 females ($p=0.07$) and vice versa (A1221 cyclic with DMSO acyclic) ($p=0.08$) (Figure 3.5B). This effect was also observed in the ME although with a weak trend ($p=0.1$ and 0.14 respectively). In the ARC 2 validated gene clusters were identified: 1) the upper cluster composed of *Gnrhr* and *Bdnf* displayed increased expression in the EB females, and 2) the lower cluster consisting of decreased expression in the A1221 acyclic vs DMSO acyclic but similar expression to cyclic DMSO females.

In the ME, the 2 validated gene clusters were distinguished by increased expression in the A1221 cyclic females (upper cluster) and differences between the DMSO cyclic females and EDC-treated acyclic females (EB acyclic and A1221 acyclic) (lower cluster, Figure 3.5C).

**Integration of genes, hormones and somatic data networks in females**

To examine the relationships among gonadal hormones, somatic changes and hypothalamic gene expression relative to reproductive cycling status in females, we used the network analysis platform Cytoscape (Kohl, 2011; Kohl et al., 2011; O'Connell and Hofmann, 2012) to generate networks based on significant Pearson correlation coefficients between hormones, endocrine tissues, and relative gene expression in each brain region. Data were first collapsed across the treatment groups in each region to identify common relationships that change during the transition from cyclicity to acyclicity. Genes with a significant interaction of treatment and cycling status in the ARC and ME are indicated with larger font ($p < 0.1$) and bolded ($p < 0.05$) to determine how/if their relationships with other genes, hormones, and somatic markers might be changing in the process to acyclicity (Figure 3.6).

Relationships in the AVPV showed the greatest difference between cyclic and acyclic females with the cyclic females displaying a number of positive and negative correlations (Figure 3.6). In the acyclic females there were few correlations, mostly negative with serum LH level. While there were not as many differences in the number of significant correlations in the ARC with reproductive aging there were differences in endpoints that serve as "hubs" of regulation. In the cyclic ARC, *Gnrh* had numerous positive correlations, 13 of the 27 were genes that were significantly altered in the ARC whereas the gonadosomatic index (GSI) and serum estradiol had mostly negative correlations. In the acyclic ARC, there were numerous positive correlations with *Gnrhr* (11/25 altered in the ARC) and pituitary weight, and numerous negative correlations with

uterine weight. Finally in the ME, a similar number of correlations were observed in both networks, with most between genes rather than with hormone or somatic endpoints. Notably, the number of positive correlations with Nr3c1 almost doubles from 8 to 14 from cyclic to acyclic.

## FIGURES AND TABLES

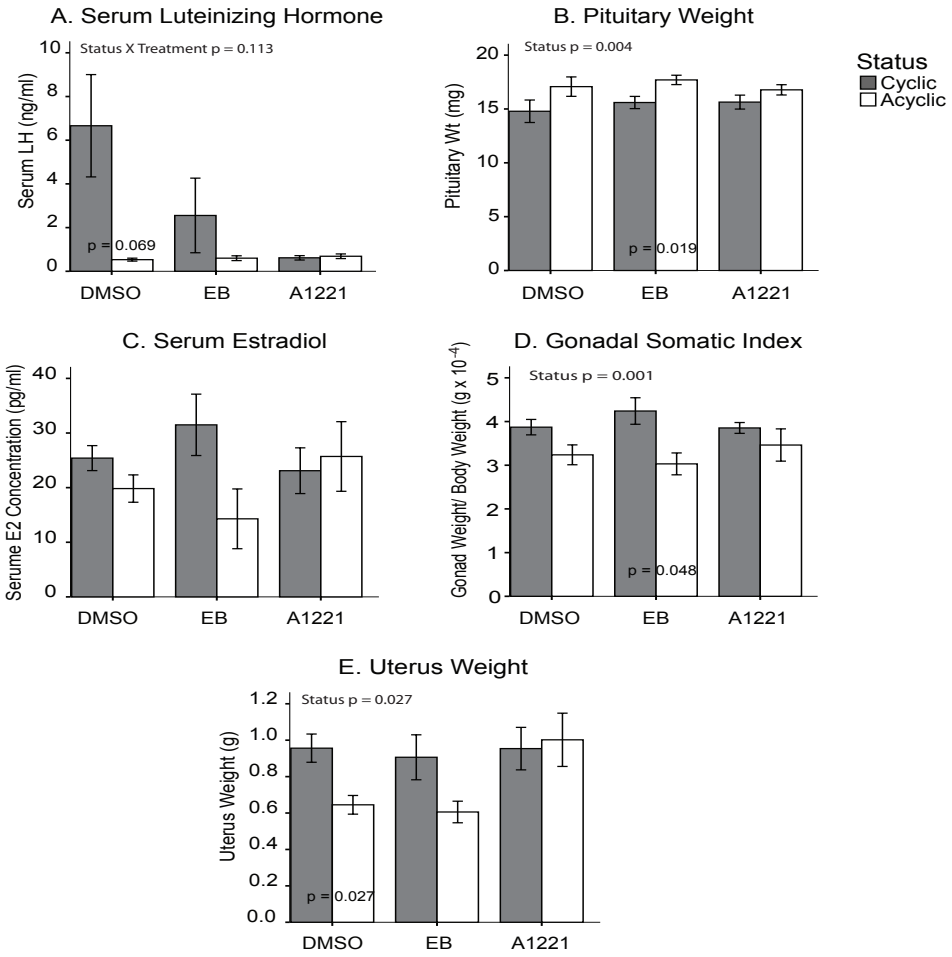



Figure 3.1: Effects of prenatal EDCs on hormones and somatic markers in aging female rats.

Females were classified as cyclic or acyclic. Significant p-values or trends for main effects of Treatment or Status, and Status x Treatment interactions, are indicated at the top of graphs. Differences between cyclic and acyclic animals within groups are indicated by p-values overlaid on relevant bar pairs. Cycle status had significant effects on pituitary weight, gonadal somatic index and uterine weights. Although not significant, serum luteinizing hormone (A) was decreased in acyclic females exposed to DMSO and EB an effect not observed in the A1221 animals. Pituitary weights (B) significantly increase in acyclic animals whereas GSI (D) and uterine weights (E) decrease in acyclic females when compared to cycling animals. No effects were observed on serum estradiol (E2) concentrations (C).

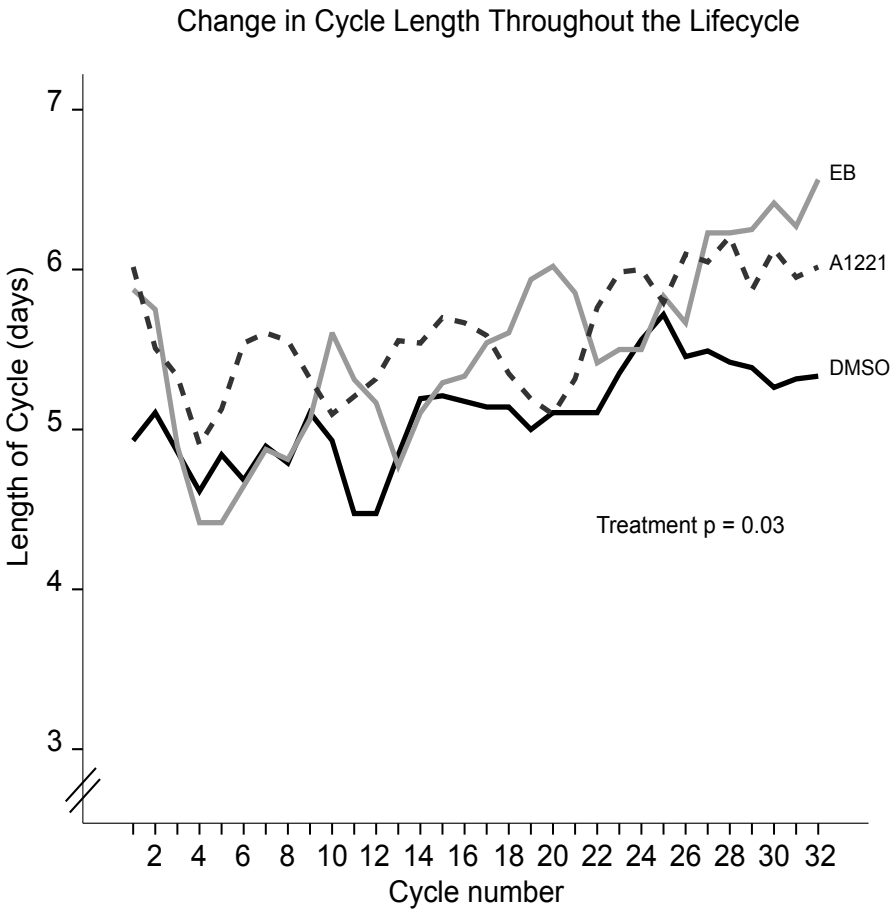



Figure 3.2: Length of estrous cycles in females treated throughout the lifecycle.

The length of each cycle was determined beginning from vaginal opening through the end of life. Data were transformed to the moving average for each animal to account for any missing data and analyzed by repeated measures ANOVA. The length of all cycles increased over time independent of treatment. Additionally, treatment significantly altered the length of estrous cycles (p=0.03). Post-hoc analysis revealed that females treated with A1221 (dashed line) had longer cycles when compared to the DMSO controls (solid black line) throughout the lifecycle (p=0.03). EB exposure (gray line) had no effect on cycle length compared to DMSO.

Figure 3.3:

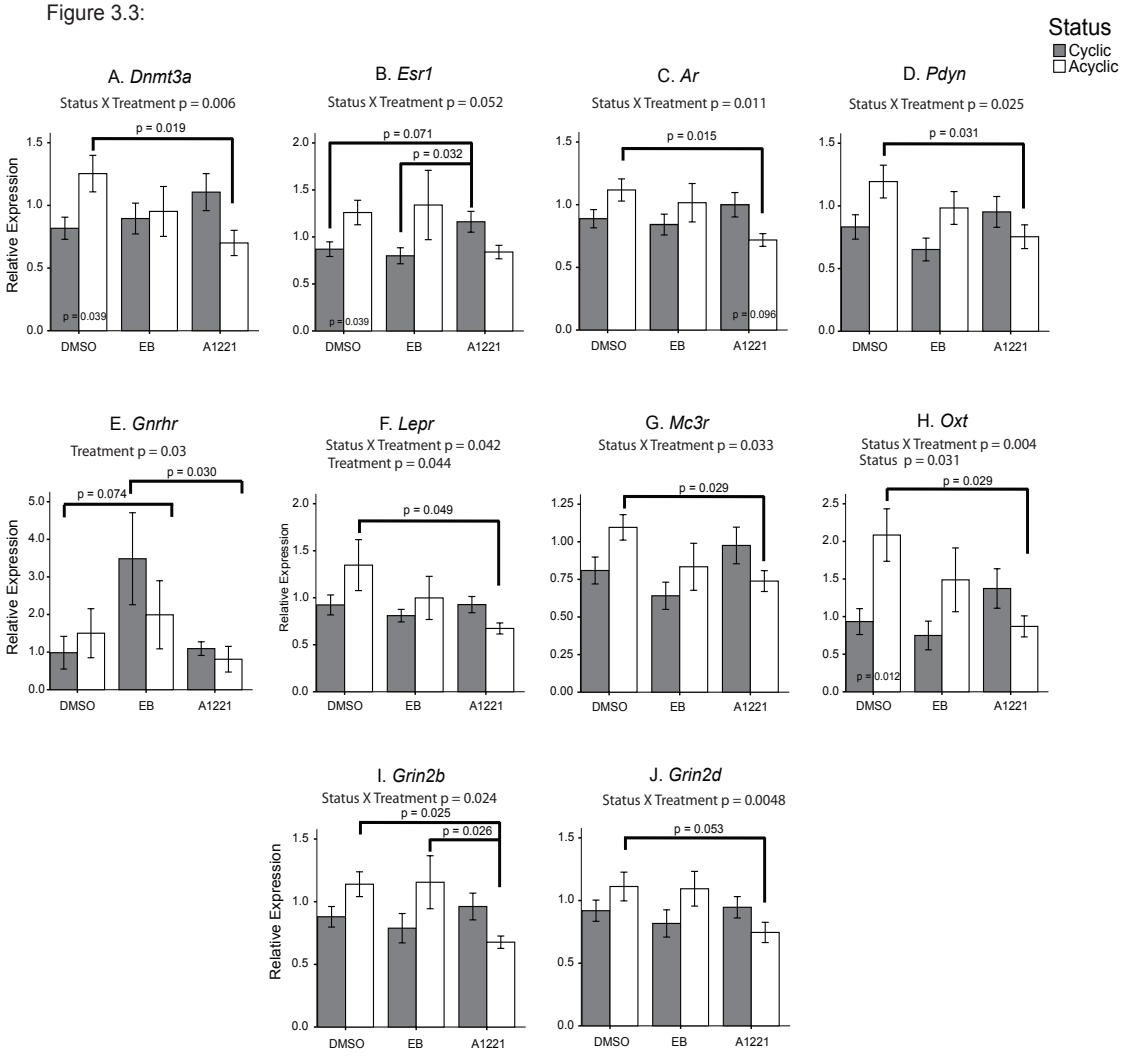



Figure 3.3: Expression of 10 genes in the ARC affected by treatment.

These genes were selected based on significant effects of Treatment, Cycle Status, or interactions of Status x Treatment. The most frequent finding was an interaction effect, with lower gene expression in the acyclic A1221 females when compared to acyclic DMSO counterparts. In addition, many genes show increased expression in DMSO acyclic compared to DMSO cyclic females, a pattern that was reversed in A1221 females.

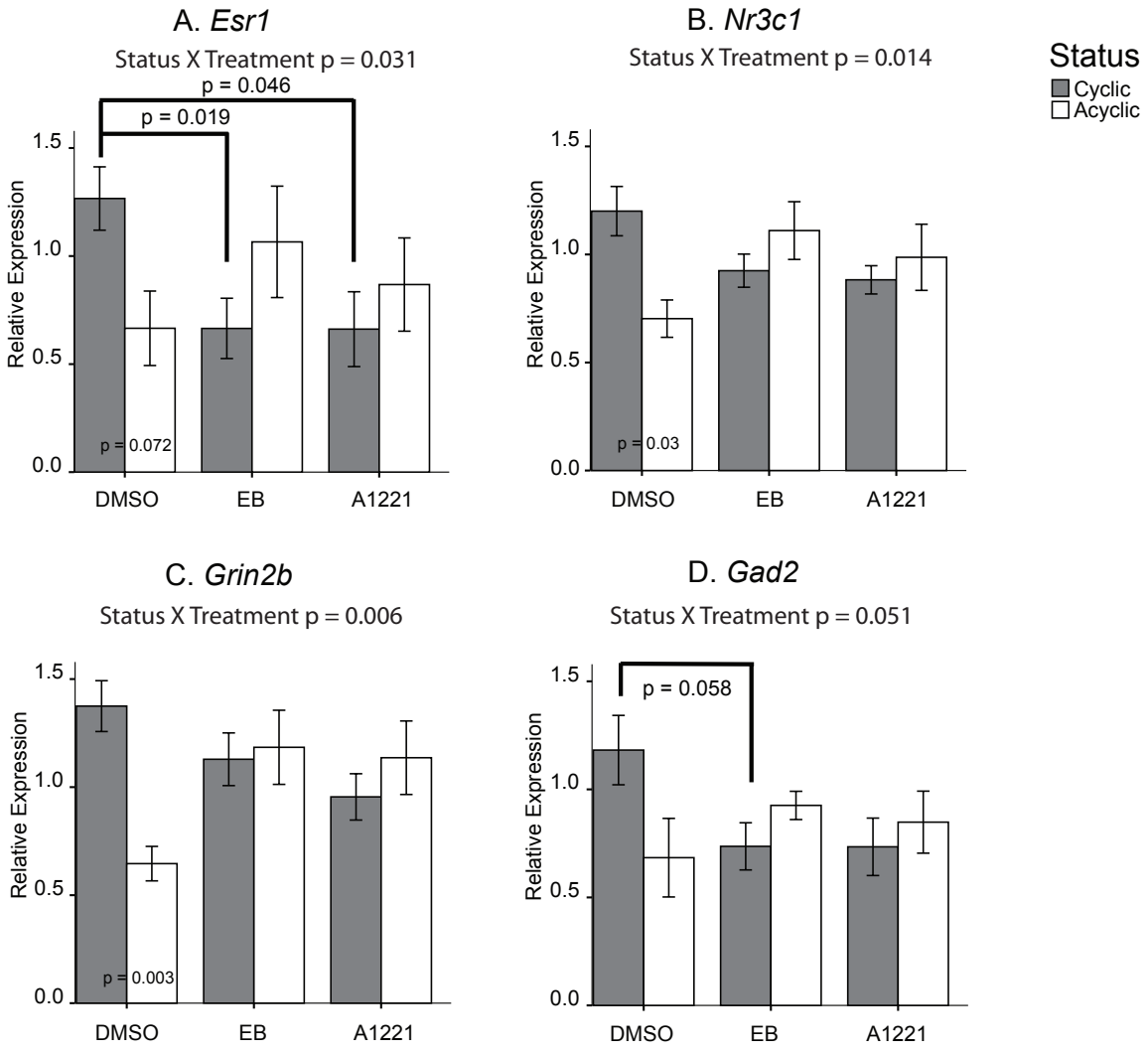



Figure 3.4: Expression of 4 genes in the ME affected by treatment.

Genes were selected based on significant effects interactions of Status x Treatment. In general, there was lower expression in the cyclic A1221 and the cyclic EB females when compared to cyclic DMSO counterparts. In DMSO females, all four genes had lower expression in acyclic than cyclic rats, an effect not seen in either A1221 or EB females.

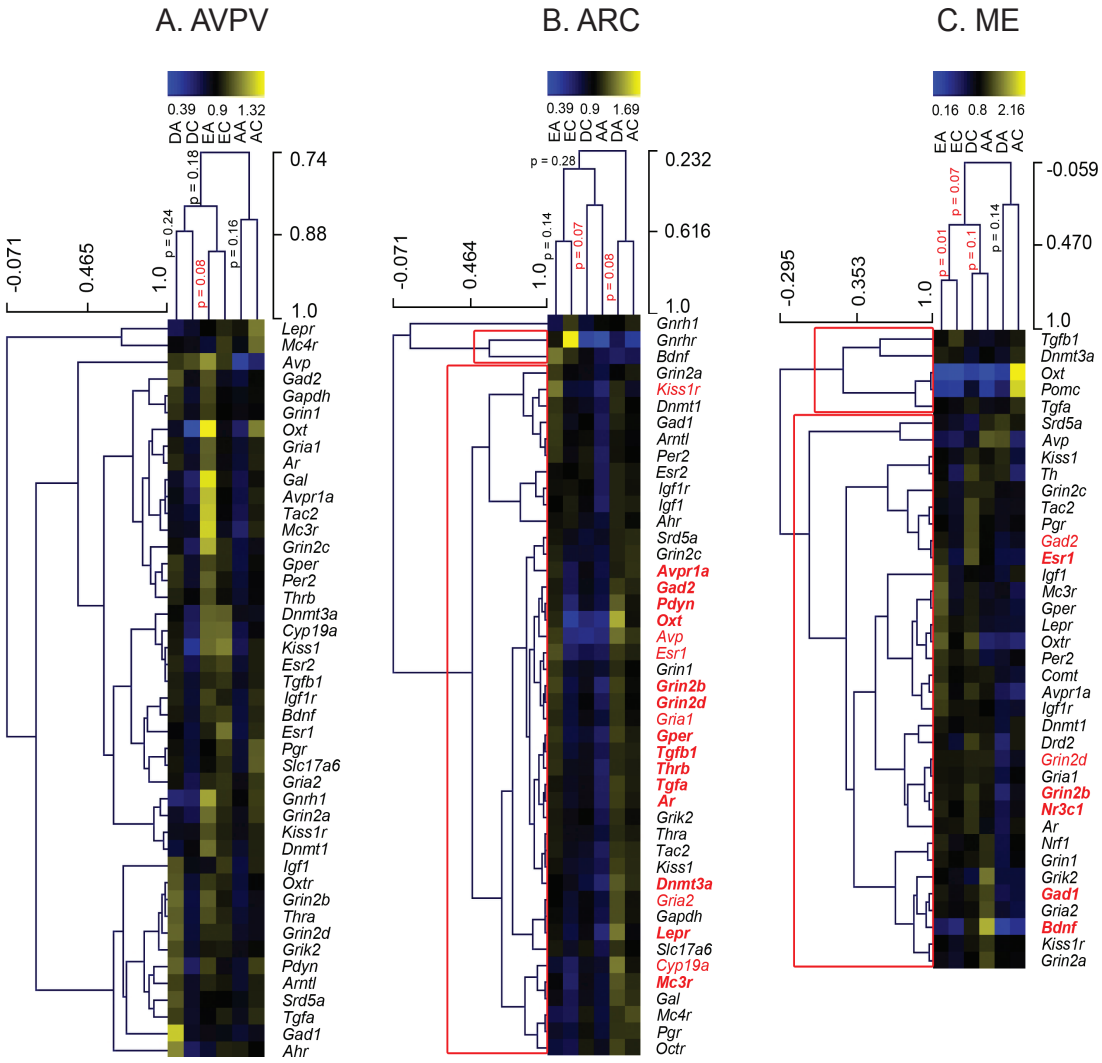



Figure 3.5: Clustergrams of gene expression in females.

Data for AVPV (A), ARC (B) and ME (C) is shown. Clusters were validated using R statistical packages and validated gene clusters are indicated by a red box. To maintain legibility of the figure, red boxes indicate the most external validated gene clusters (side) whereas, p-values are indicated for clusters by treatment and cycle status (top). Genes with a Status x Treatment interaction are indicated in red text ($p < 0.1$) and bolded ($p < 0.05$) to identify gene clusters associated with specific status/treatment related expression patterns. This effect was observed in the ARC where all significantly altered genes are in one cluster. The p-value from the validation is listed for the clusters of the groups. Abbreviations: DC, DMSO cyclic; DA, DMSO acyclic; EC, EB cyclic; EA, EB acyclic; AC, A1221 cyclic; AA, A1221 acyclic.

Figure 3.6

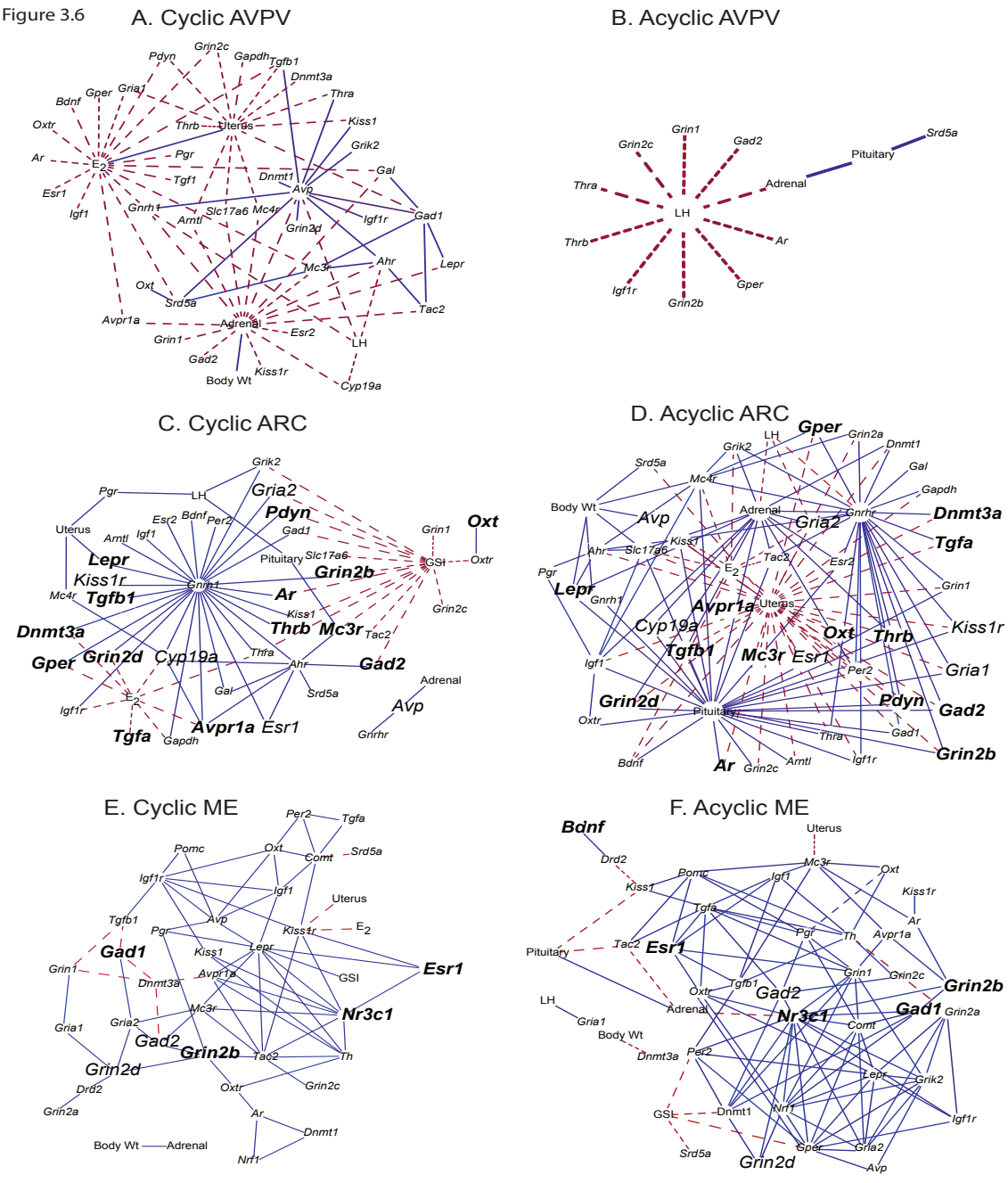



Figure 3.6: Cyctoscape networks of genes, hormones and somatic changes

Data are presented for 3 brain regions involved in reproductive function (AVPV, ARC and ME) in cyclic vs acyclic females. Genes with a significant interaction of treatment X status are indicated by larger font ($p < 0.1$) and bolded ($p < 0.05$). Overall results show that the networks differ profoundly between cyclic and acyclic rats in each brain region.

| Gene Symbol | Gene Name | p-value Status | p-value Treatment | p-value Interactions | DMSO Cyclic | DMSO Acyclic | EB Cyclic | EB Acyclic | A1221 Cyclic | A1221 Acyclic |
|---|---|---|---|---|---|---|---|---|---|---|
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.848 | 0.932 | 0.535 | 0.98 (+/-0.11) | 0.91 (+/-0.09) | 0.86 (+/-0.09) | 1.01 (+/-0.13) | 0.98 (+/-0.06) | 0.96 (+/-0.08) |
| *Ahr* | aryl hydrocarbon receptor | 0.88 | 0.392 | 0.712 | 1.06 (+/-0.19) | 1.70 (+/-0.57) | 1.45 (+/-0.23) | 1.20 (+/-0.22) | 1.36 (+/-0.16) | 1.43 (+/-0.37) |
| *Ar* | androgen receptor | 0.571 | 0.753 | 0.286 | 0.85 (+/-0.1) | 1.00 (+/-0.17) | 0.91 (+/-0.08) | 1.10 (+/-0.11) | 0.99 (+/-0.12) | 0.82 (+/-0.14) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.792 | 0.818 | 0.299 | 0.96 (+/-0.1) | 1.19 (+/-0.2) | 1.10 (+/-0.09) | 1.13 (+/-0.06) | 1.12 (+/-0.13) | 0.95 (+/-0.14) |
| *Avp* | arginine vasopressin | 0.963 | 0.413 | 0.932 | 3.36 (+/-1.33) | 3.24 (+/-1.56) | 3.24 (+/-1.5) | 3.74 (+/-1.98) | 2.17 (+/-0.56) | 1.64 (+/-0.67) |
| *Avpr1a* | arginine vasopressin receptor 1A | 0.426 | 0.108 | 0.127 | 0.85 (+/-0.1) | 1.01 (+/-0.18) | 1.07 (+/-0.14) | 1.43 (+/-0.25) | 1.13 (+/-0.13) | 0.89 (+/-0.14) |
| *Bdnf* | brain-derived neurotrophic factor | 0.849 | 0.606 | 0.494 | 0.89 (+/-0.12) | 1.04 (+/-0.24) | 1.11 (+/-0.13) | 1.09 (+/-0.14) | 1.06 (+/-0.12) | 0.86 (+/-0.16) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.801 | 0.223 | 0.505 | 0.92 (+/-0.15) | 1.23 (+/-0.29) | 1.42 (+/-0.22) | 1.42 (+/-0.29) | 1.16 (+/-0.18) | 0.99 (+/-0.19) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.647 | 0.224 | 0.436 | 1.00 (+/-0.1) | 0.89 (+/-0.18) | 1.10 (+/-0.07) | 1.24 (+/-0.12) | 1.09 (+/-0.12) | 0.92 (+/-0.15) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.507 | 0.159 | 0.709 | 0.86 (+/-0.11) | 1.10 (+/-0.2) | 1.27 (+/-0.12) | 1.28 (+/-0.22) | 1.01 (+/-0.12) | 1.02 (+/-0.19) |
| *Esr1* | estrogen receptor 1 | 0.587 | 0.562 | 0.586 | 0.90 (+/-0.16) | 1.04 (+/-0.25) | 1.27 (+/-0.2) | 1.11 (+/-0.25) | 1.13 (+/-0.15) | 0.89 (+/-0.18) |
| *Esr2* | estrogen receptor 2 | 0.905 | 0.519 | 0.354 | 0.80 (+/-0.1) | 1.04 (+/-0.17) | 1.10 (+/-0.14) | 1.07 (+/-0.2) | 1.02 (+/-0.13) | 0.86 (+/-0.14) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.682 | 0.765 | 0.272 | 1.22 (+/-0.23) | 1.82 (+/-0.42) | 1.26 (+/-0.11) | 1.27 (+/-0.15) | 1.24 (+/-0.1) | 1.17 (+/-0.2) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.28 | 0.474 | 0.136 | 0.91 (+/-0.07) | 1.12 (+/-0.16) | 0.89 (+/-0.05) | 1.11 (+/-0.09) | 0.97 (+/-0.1) | 0.83 (+/-0.12) |
| *Gal* | galanin prepropeptide | 0.391 | ***0.074*** | 0.226 | 0.83 (+/-0.12) | 1.01 (+/-0.22) | 1.15 (+/-0.16) | 1.58 (+/-0.31) | 1.14 (+/-0.17) | 0.92 (+/-0.17) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.412 | 0.866 | 0.59 | 0.92 (+/-0.12) | 1.14 (+/-0.24) | 1.02 (+/-0.09) | 1.17 (+/-0.11) | 1.05 (+/-0.13) | 0.98 (+/-0.18) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.933 | ***0.088*** | 0.692 | 0.81 (+/-0.11) | 0.76 (+/-0.23) | 1.16 (+/-0.24) | 1.38 (+/-0.31) | 1.18 (+/-0.17) | 1.05 (+/-0.25) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.78 | 0.487 | 0.373 | 0.84 (+/-0.09) | 1.03 (+/-0.18) | 1.01 (+/-0.12) | 1.06 (+/-0.18) | 0.95 (+/-0.1) | 0.80 (+/-0.12) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.586 | 0.499 | 0.278 | 0.82 (+/-0.09) | 0.97 (+/-0.16) | 0.95 (+/-0.07) | 1.10 (+/-0.1) | 0.98 (+/-0.11) | 0.83 (+/-0.14) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.93 | 0.406 | 0.294 | 0.90 (+/-0.09) | 1.12 (+/-0.16) | 1.20 (+/-0.1) | 1.16 (+/-0.13) | 1.20 (+/-0.11) | 1.04 (+/-0.16) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.545 | 0.866 | 0.361 | 1.01 (+/-0.13) | 1.30 (+/-0.23) | 1.20 (+/-0.08) | 1.19 (+/-0.12) | 1.16 (+/-0.09) | 1.08 (+/-0.16) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.353 | 0.75 | 0.669 | 0.96 (+/-0.08) | 1.12 (+/-0.17) | 1.04 (+/-0.07) | 1.15 (+/-0.09) | 1.02 (+/-0.11) | 1.00 (+/-0.11) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.899 | 0.206 | 0.511 | 0.86 (+/-0.1) | 0.95 (+/-0.18) | 1.12 (+/-0.12) | 1.26 (+/-0.19) | 1.16 (+/-0.13) | 0.98 (+/-0.18) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.235 | 0.267 | 0.199 | 0.80 (+/-0.1) | 1.12 (+/-0.18) | 0.97 (+/-0.08) | 1.10 (+/-0.12) | 0.89 (+/-0.1) | 0.79 (+/-0.14) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.23 | **0.046** | 0.316 | 0.85 (+/-0.1) | 1.10 (+/-0.19) | 1.17 (+/-0.14) | 1.43 (+/-0.14) | 1.02 (+/-0.12) | 0.92 (+/-0.16) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.407 | 0.529 | 0.387 | 0.80 (+/-0.11) | 1.10 (+/-0.22) | 1.00 (+/-0.08) | 1.00 (+/-0.15) | 0.87 (+/-0.1) | 0.84 (+/-0.13) |
| *Igf1* | insulin-like growth factor 1 | 0.651 | 0.807 | 0.335 | 0.84 (+/-0.11) | 1.01 (+/-0.22) | 0.97 (+/-0.13) | 0.83 (+/-0.13) | 0.93 (+/-0.1) | 0.75 (+/-0.11) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.905 | 0.603 | 0.242 | 0.93 (+/-0.12) | 1.12 (+/-0.21) | 1.11 (+/-0.08) | 1.19 (+/-0.12) | 1.15 (+/-0.1) | 0.92 (+/-0.14) |
| *Kiss1* | KiSS-1 metastasis-suppressor | 0.961 | 0.301 | 0.434 | 1.00 (+/-0.2) | 1.69 (+/-0.42) | 2.00 (+/-0.42) | 1.94 (+/-0.73) | 1.70 (+/-0.25) | 1.31 (+/-0.36) |
| *Kiss1r* | KISS1 receptor | 0.916 | 0.518 | 0.764 | 0.97 (+/-0.08) | 0.95 (+/-0.19) | 1.08 (+/-0.11) | 1.17 (+/-0.1) | 1.08 (+/-0.14) | 0.97 (+/-0.18) |
| *Lepr* | leptin receptor | 0.299 | ***0.099*** | 0.875 | 0.98 (+/-0.07) | 0.88 (+/-0.12) | 1.16 (+/-0.12) | 1.09 (+/-0.19) | 1.33 (+/-0.15) | 1.13 (+/-0.19) |
| *Mc3r* | melanocortin 3 receptor | 0.721 | 0.317 | 0.102 | 0.82 (+/-0.13) | 0.84 (+/-0.22) | 0.83 (+/-0.12) | 1.24 (+/-0.18) | 0.96 (+/-0.15) | 0.67 (+/-0.12) |
| *Mc4r* | melanocortin 4 receptor | 0.267 | 0.753 | 0.619 | 0.98 (+/-0.15) | 1.01 (+/-0.2) | 1.15 (+/-0.17) | 0.90 (+/-0.14) | 1.24 (+/-0.15) | 0.99 (+/-0.17) |
| *Oxt* | oxytocin, prepropeptide | 0.206 | 0.209 | 0.114 | 0.75 (+/-0.14) | 1.55 (+/-0.27) | 1.62 (+/-0.45) | 2.49 (+/-0.76) | 2.09 (+/-0.65) | 1.29 (+/-0.36) |
| *Oxtr* | oxytocin receptor | 0.538 | 0.492 | 0.259 | 0.89 (+/-0.1) | 1.18 (+/-0.16) | 1.08 (+/-0.12) | 1.12 (+/-0.1) | 1.00 (+/-0.11) | 0.88 (+/-0.15) |
| *Pdyn* | prodynorphin | 0.393 | 0.93 | 0.132 | 0.80 (+/-0.14) | 1.23 (+/-0.21) | 1.00 (+/-0.13) | 1.17 (+/-0.23) | 1.17 (+/-0.17) | 0.93 (+/-0.14) |
| *Per2* | period homolog 2 | 0.548 | 0.521 | 0.531 | 0.87 (+/-0.1) | 1.06 (+/-0.21) | 1.01 (+/-0.14) | 1.15 (+/-0.18) | 0.96 (+/-0.13) | 0.85 (+/-0.15) |
| *Pgr* | progesterone receptor | 0.511 | 0.798 | 0.368 | 0.83 (+/-0.12) | 0.97 (+/-0.19) | 1.06 (+/-0.14) | 0.95 (+/-0.12) | 1.11 (+/-0.13) | 0.84 (+/-0.15) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.605 | 0.807 | 0.288 | 0.86 (+/-0.12) | 1.03 (+/-0.19) | 1.07 (+/-0.15) | 1.01 (+/-0.09) | 1.16 (+/-0.13) | 0.87 (+/-0.15) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.703 | 0.972 | 0.511 | 1.08 (+/-0.19) | 1.37 (+/-0.28) | 1.18 (+/-0.11) | 1.19 (+/-0.18) | 1.25 (+/-0.14) | 1.12 (+/-0.2) |
| *Tac2* | tachykinin 2 | 0.605 | 0.221 | 0.216 | 0.90 (+/-0.12) | 0.96 (+/-0.16) | 1.03 (+/-0.16) | 1.42 (+/-0.29) | 1.09 (+/-0.18) | 0.86 (+/-0.13) |
| *Tgfa* | transforming growth factor, alpha | 0.99 | 0.993 | 0.374 | 0.86 (+/-0.12) | 1.07 (+/-0.19) | 1.01 (+/-0.09) | 0.95 (+/-0.13) | 1.04 (+/-0.11) | 0.89 (+/-0.16) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.976 | 0.708 | 0.559 | 0.97 (+/-0.13) | 1.14 (+/-0.24) | 1.19 (+/-0.12) | 1.16 (+/-0.11) | 1.14 (+/-0.1) | 1.00 (+/-0.17) |
| *Thra* | thyroid hormone receptor, alpha | 0.297 | 0.351 | 0.346 | 0.87 (+/-0.11) | 1.20 (+/-0.19) | 1.09 (+/-0.11) | 1.17 (+/-0.1) | 0.96 (+/-0.11) | 0.90 (+/-0.16) |
| *Thrb* | thyroid hormone receptor, beta | 0.729 | 0.522 | 0.663 | 1.03 (+/-0.09) | 1.11 (+/-0.2) | 1.07 (+/-0.1) | 1.20 (+/-0.12) | 1.03 (+/-0.11) | 0.94 (+/-0.16) |

Table 3.1: Gene Expression in the Female Anteroventral Periventricular Nucleus (AVPV)

Relative gene expression for each treatment group is shown. P-values are listed for each variable. Significant main effects of treatment and status, or interaction of treatment x status are bolded ($p < 0.05$). Italics indicated that there was a trend ($p < 0.1$). Non-detectable genes are excluded from the table (*Dnmt3l*, *Gnrhr*, *Mc5r*).

| Gene Symbol | Gene Name | p-value Status | p-value Treatment | p-value Interactions | DMSO Cyclic | DMSO Acyclic | EB Cyclic | EB Acyclic | A1221 Cyclic | A1221 Acyclic |
|---|---|---|---|---|---|---|---|---|---|---|
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.788 | 0.99 | 0.375 | 1.05 (+/-0.06) | 0.94 (+/-0.14) | 0.96 (+/-0.1) | 1.01 (+/-0.1) | 0.92 (+/-0.05) | 1.04 (+/-0.08) |
| *Ahr* | aryl hydrocarbon receptor | 0.991 | 0.358 | 0.608 | 0.96 (+/-0.08) | 1.16 (+/-0.16) | 1.11 (+/-0.17) | 1.07 (+/-0.17) | 1.05 (+/-0.21) | 0.90 (+/-0.2) |
| *Ar* | androgen receptor | 0.595 | 0.281 | **0.011** | 0.89 (+/-0.07) | 1.12 (+/-0.09) | 0.84 (+/-0.08) | 1.02 (+/-0.15) | 1.00 (+/-0.1) | 0.72 (+/-0.05) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.814 | ***0.088*** | 0.113 | 1.04 (+/-0.07) | 1.06 (+/-0.05) | 0.98 (+/-0.08) | 1.19 (+/-0.18) | 0.99 (+/-0.08) | 0.81 (+/-0.09) |
| *Avp* | arginine vasopressin | 0.13 | 0.36 | ***0.097*** | 0.85 (+/-0.14) | 1.78 (+/-0.34) | 0.88 (+/-0.34) | 1.54 (+/-0.49) | 1.51 (+/-0.32) | 0.90 (+/-0.17) |
| *Avpr1a* | arginine vasopressin receptor 1A | 0.675 | 0.929 | **0.034** | 0.90 (+/-0.1) | 1.03 (+/-0.08) | 0.75 (+/-0.1) | 1.07 (+/-0.27) | 1.10 (+/-0.11) | 0.78 (+/-0.09) |
| *Bdnf* | brain-derived neurotrophic factor | 0.918 | ***0.085*** | 0.605 | 1.25 (+/-0.19) | 0.99 (+/-0.17) | 1.50 (+/-0.39) | 1.81 (+/-0.47) | 1.05 (+/-0.14) | 1.06 (+/-0.28) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.248 | 0.105 | ***0.097*** | 0.89 (+/-0.09) | 1.36 (+/-0.35) | 0.74 (+/-0.09) | 0.85 (+/-0.12) | 1.00 (+/-0.11) | 0.86 (+/-0.09) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.399 | 0.397 | 0.188 | 0.95 (+/-0.1) | 1.19 (+/-0.11) | 1.00 (+/-0.14) | 1.21 (+/-0.26) | 1.03 (+/-0.09) | 0.85 (+/-0.12) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.795 | 0.545 | **0.006** | 0.82 (+/-0.09) | 1.25 (+/-0.15) | 0.90 (+/-0.12) | 0.95 (+/-0.2) | 1.11 (+/-0.15) | 0.70 (+/-0.1) |
| *Esr1* | estrogen receptor 1 | 0.294 | 0.642 | ***0.052*** | 0.87 (+/-0.08) | 1.26 (+/-0.13) | 0.80 (+/-0.09) | 1.34 (+/-0.37) | 1.16 (+/-0.11) | 0.84 (+/-0.07) |
| *Esr2* | estrogen receptor 2 | 0.511 | 0.318 | 0.291 | 1.01 (+/-0.09) | 1.05 (+/-0.15) | 0.94 (+/-0.15) | 0.97 (+/-0.12) | 1.00 (+/-0.07) | 0.74 (+/-0.06) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.62 | 0.311 | 0.115 | 0.92 (+/-0.1) | 1.03 (+/-0.08) | 0.86 (+/-0.1) | 1.11 (+/-0.22) | 0.94 (+/-0.09) | 0.73 (+/-0.08) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.35 | 0.524 | **0.022** | 0.87 (+/-0.07) | 1.10 (+/-0.09) | 0.74 (+/-0.09) | 0.99 (+/-0.19) | 1.03 (+/-0.1) | 0.78 (+/-0.09) |
| *Gal* | galanin prepropeptide | 0.674 | 0.448 | 0.122 | 0.89 (+/-0.11) | 1.19 (+/-0.12) | 0.76 (+/-0.13) | 0.90 (+/-0.22) | 1.10 (+/-0.18) | 0.82 (+/-0.12) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.521 | 0.317 | 0.122 | 0.87 (+/-0.1) | 1.12 (+/-0.09) | 0.78 (+/-0.11) | 0.91 (+/-0.2) | 0.94 (+/-0.1) | 0.74 (+/-0.11) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.565 | 0.836 | 0.6 | 0.81 (+/-0.09) | 0.93 (+/-0.24) | 1.11 (+/-0.23) | 0.77 (+/-0.28) | 1.03 (+/-0.22) | 0.95 (+/-0.23) |
| *Gnrhr* | gonadotropin-releasing hormone receptor | 0.44 | **0.03** | 0.335 | 0.98 (+/-0.44) | 1.50 (+/-0.65) | 3.48 (+/-1.22) | 1.99 (+/-0.91) | 1.09 (+/-0.18) | 0.81 (+/-0.34) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.293 | 0.568 | **0.015** | 0.99 (+/-0.09) | 1.36 (+/-0.18) | 0.94 (+/-0.12) | 1.31 (+/-0.2) | 1.21 (+/-0.12) | 0.85 (+/-0.17) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.221 | 0.505 | ***0.094*** | 0.88 (+/-0.07) | 1.08 (+/-0.1) | 0.79 (+/-0.08) | 1.03 (+/-0.18) | 0.94 (+/-0.08) | 0.80 (+/-0.09) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.394 | 0.171 | ***0.055*** | 0.91 (+/-0.08) | 1.19 (+/-0.14) | 0.84 (+/-0.08) | 0.95 (+/-0.14) | 0.98 (+/-0.08) | 0.81 (+/-0.07) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.816 | 0.25 | 0.128 | 1.06 (+/-0.1) | 1.23 (+/-0.13) | 1.01 (+/-0.09) | 1.14 (+/-0.15) | 1.08 (+/-0.1) | 0.86 (+/-0.06) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.179 | 0.641 | 0.161 | 0.96 (+/-0.09) | 1.18 (+/-0.12) | 0.92 (+/-0.12) | 1.22 (+/-0.21) | 1.04 (+/-0.09) | 0.91 (+/-0.11) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.957 | 0.683 | 0.29 | 1.12 (+/-0.13) | 1.16 (+/-0.14) | 1.14 (+/-0.2) | 1.40 (+/-0.27) | 1.27 (+/-0.13) | 0.99 (+/-0.16) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.245 | 0.306 | **0.024** | 0.88 (+/-0.08) | 1.14 (+/-0.1) | 0.79 (+/-0.12) | 1.16 (+/-0.21) | 0.96 (+/-0.11) | 0.68 (+/-0.05) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.732 | 0.995 | 0.13 | 0.86 (+/-0.08) | 1.06 (+/-0.12) | 0.85 (+/-0.14) | 1.06 (+/-0.2) | 1.11 (+/-0.18) | 0.82 (+/-0.08) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.289 | 0.226 | **0.048** | 0.92 (+/-0.08) | 1.11 (+/-0.11) | 0.82 (+/-0.11) | 1.09 (+/-0.14) | 0.95 (+/-0.09) | 0.75 (+/-0.08) |
| *Igf1* | insulin-like growth factor 1 | 0.452 | 0.631 | 0.252 | 1.00 (+/-0.1) | 1.15 (+/-0.15) | 1.12 (+/-0.16) | 1.03 (+/-0.16) | 1.12 (+/-0.18) | 0.78 (+/-0.13) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.514 | 0.254 | 0.165 | 0.99 (+/-0.08) | 1.10 (+/-0.11) | 1.03 (+/-0.09) | 1.02 (+/-0.15) | 1.02 (+/-0.1) | 0.77 (+/-0.09) |
| *Kiss1* | KiSS-1 metastasis-suppressor | 0.806 | 0.596 | 0.225 | 0.91 (+/-0.06) | 1.26 (+/-0.19) | 0.93 (+/-0.18) | 1.09 (+/-0.32) | 1.18 (+/-0.16) | 0.84 (+/-0.11) |
| *Kiss1r* | KISS1 receptor | ***0.085*** | ***0.093*** | ***0.057*** | 0.92 (+/-0.1) | 1.21 (+/-0.12) | 0.93 (+/-0.19) | 1.50 (+/-0.32) | 0.96 (+/-0.12) | 0.77 (+/-0.11) |
| *Lepr* | leptin receptor | 0.304 | **0.044** | **0.042** | 0.92 (+/-0.11) | 1.35 (+/-0.27) | 0.81 (+/-0.07) | 1.00 (+/-0.23) | 0.93 (+/-0.09) | 0.67 (+/-0.06) |
| *Mc3r* | melanocortin 3 receptor | 0.372 | 0.184 | **0.033** | 0.81 (+/-0.09) | 1.10 (+/-0.08) | 0.64 (+/-0.09) | 0.83 (+/-0.16) | 0.98 (+/-0.12) | 0.74 (+/-0.07) |
| *Mc4r* | melanocortin 4 receptor | 0.519 | 0.23 | 0.313 | 1.14 (+/-0.16) | 1.24 (+/-0.18) | 0.85 (+/-0.15) | 0.88 (+/-0.12) | 1.33 (+/-0.18) | 0.91 (+/-0.15) |
| *Oxt* | oxytocin, prepropeptide | **0.031** | 0.206 | **0.004** | 0.93 (+/-0.17) | 2.08 (+/-0.35) | 0.75 (+/-0.19) | 1.49 (+/-0.42) | 1.37 (+/-0.26) | 0.87 (+/-0.14) |
| *Oxtr* | oxytocin receptor | 1 | 0.102 | 0.478 | 0.98 (+/-0.1) | 1.11 (+/-0.15) | 0.74 (+/-0.12) | 0.78 (+/-0.09) | 1.00 (+/-0.14) | 0.84 (+/-0.1) |
| *Pdyn* | prodynorphin | ***0.088*** | 0.214 | **0.025** | 0.83 (+/-0.1) | 1.19 (+/-0.13) | 0.65 (+/-0.09) | 0.98 (+/-0.13) | 0.95 (+/-0.12) | 0.75 (+/-0.09) |
| *Per2* | period homolog 2 | 0.914 | 0.311 | 0.303 | 1.03 (+/-0.11) | 1.01 (+/-0.11) | 0.92 (+/-0.12) | 1.13 (+/-0.2) | 0.96 (+/-0.13) | 0.74 (+/-0.12) |
| *Pgr* | progesterone receptor | 0.64 | 0.168 | 0.109 | 0.96 (+/-0.06) | 1.08 (+/-0.09) | 0.80 (+/-0.11) | 0.84 (+/-0.11) | 1.09 (+/-0.12) | 0.81 (+/-0.08) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.792 | 0.309 | 0.387 | 0.98 (+/-0.11) | 1.07 (+/-0.13) | 0.83 (+/-0.14) | 0.99 (+/-0.14) | 0.92 (+/-0.13) | 0.75 (+/-0.09) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.654 | 0.95 | 0.121 | 0.93 (+/-0.08) | 1.18 (+/-0.13) | 0.99 (+/-0.14) | 1.13 (+/-0.17) | 1.21 (+/-0.14) | 0.96 (+/-0.1) |
| *Tac2* | tachykinin 2 | 0.851 | 0.867 | 0.132 | 0.98 (+/-0.1) | 1.28 (+/-0.22) | 1.01 (+/-0.2) | 1.19 (+/-0.35) | 1.23 (+/-0.16) | 0.85 (+/-0.14) |
| *Tgfa* | transforming growth factor, alpha | 0.8 | 0.439 | **0.042** | 0.91 (+/-0.08) | 1.15 (+/-0.18) | 0.88 (+/-0.11) | 1.01 (+/-0.18) | 1.04 (+/-0.1) | 0.73 (+/-0.09) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.514 | 0.79 | **0.044** | 0.91 (+/-0.07) | 1.12 (+/-0.12) | 0.85 (+/-0.09) | 1.12 (+/-0.21) | 1.08 (+/-0.13) | 0.79 (+/-0.12) |
| *Thra* | thyroid hormone receptor, alpha | 0.671 | 0.836 | 0.183 | 0.87 (+/-0.09) | 1.06 (+/-0.09) | 0.88 (+/-0.12) | 1.01 (+/-0.16) | 1.00 (+/-0.1) | 0.80 (+/-0.13) |
| *Thrb* | thyroid hormone receptor, beta | 0.752 | 0.503 | **0.033** | 0.87 (+/-0.07) | 1.06 (+/-0.1) | 0.86 (+/-0.11) | 1.07 (+/-0.19) | 1.01 (+/-0.11) | 0.70 (+/-0.1) |

Table 3.2: Gene Expression in the Female Arcuate Nucleus (ARC)

Relative gene expression for each treatment group is shown. P-values are listed for each variable. Significant main effects of treatment and status, or interaction of treatment x status are bolded ($p < 0.05$). Italics indicated that there was a trend ($p < 0.1$). Non-detectable genes are excluded from the table (*Dnmt3l*, *Mc5r*).

| Gene Symbol | Gene Name | p-value Status | p-value Treatment | p-value Interactions | DMSO Cyclic | DMSO Acyclic | EB Cyclic | EB Acyclic | A1221 Cyclic | A1221 Acyclic |
|---|---|---|---|---|---|---|---|---|---|---|
| *18S* | Eukaryotic 18S rRNA | 0.567 | 0.893 | 0.778 | 1.52 (+/-0.54) | 1.10 (+/-0.2) | 1.17 (+/-0.16) | 1.22 (+/-0.33) | 1.21 (+/-0.21) | 1.10 (+/-0.2) |
| *Ar* | androgen receptor | ***0.086*** | 0.855 | 0.288 | 1.14 (+/-0.11) | 0.66 (+/-0.13) | 0.98 (+/-0.13) | 0.97 (+/-0.23) | 0.98 (+/-0.09) | 0.83 (+/-0.15) |
| *Avp* | arginine vasopressin | 0.204 | 0.654 | 0.686 | 1.34 (+/-0.52) | 2.32 (+/-1.01) | 1.10 (+/-0.34) | 1.19 (+/-0.59) | 1.00 (+/-0.38) | 2.41 (+/-0.92) |
| *Avpr1a* | arginine vasopressin receptor 1A | 0.371 | 0.325 | 0.126 | 1.56 (+/-0.37) | 0.87 (+/-0.14) | 1.17 (+/-0.24) | 1.68 (+/-0.22) | 0.73 (+/-0.11) | 1.32 (+/-0.23) |
| *Bdnf* | brain-derived neurotrophic factor | 0.808 | 0.271 | **0.027** | 2.21 (+/-1.1) | 0.65 (+/-0.29) | 1.11 (+/-0.25) | 1.31 (+/-0.3) | 0.92 (+/-0.39) | 3.84 (+/-1.41) |
| *Comt* | catechol-O-methyltransferase | 0.575 | 0.742 | 0.435 | 1.25 (+/-0.23) | 0.99 (+/-0.15) | 1.15 (+/-0.24) | 1.42 (+/-0.41) | 0.94 (+/-0.08) | 1.29 (+/-0.19) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.508 | 0.368 | 0.143 | 1.09 (+/-0.06) | 0.80 (+/-0.1) | 0.97 (+/-0.09) | 1.12 (+/-0.05) | 1.12 (+/-0.14) | 1.08 (+/-0.15) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3 alpha | 0.769 | ***0.086*** | 0.498 | 0.94 (+/-0.1) | 0.98 (+/-0.11) | 1.22 (+/-0.11) | 1.32 (+/-0.28) | 1.40 (+/-0.15) | 1.15 (+/-0.16) |
| *Drd2* | dopamine receptor D2 | 0.334 | 0.992 | 0.21 | 1.42 (+/-0.24) | 0.65 (+/-0.17) | 0.86 (+/-0.17) | 1.12 (+/-0.23) | 1.19 (+/-0.39) | 1.19 (+/-0.41) |
| *Esr1* | estrogen receptor 1 | 0.989 | 0.584 | **0.031** | 1.27 (+/-0.15) | 0.67 (+/-0.17) | 0.67 (+/-0.14) | 1.07 (+/-0.26) | 0.66 (+/-0.17) | 0.87 (+/-0.22) |
| *Gad1* | glutamate decarboxylase 1 | 0.938 | 0.586 | **0.048** | 1.40 (+/-0.21) | 0.93 (+/-0.26) | 1.18 (+/-0.09) | 1.40 (+/-0.14) | 1.00 (+/-0.22) | 1.73 (+/-0.4) |
| *Gad2* | glutamate decarboxylase 2 | 0.595 | 0.613 | ***0.051*** | 1.18 (+/-0.16) | 0.68 (+/-0.18) | 0.74 (+/-0.11) | 0.93 (+/-0.07) | 0.73 (+/-0.13) | 0.85 (+/-0.14) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.609 | 0.978 | 0.324 | 1.18 (+/-0.45) | 1.47 (+/-0.26) | 1.34 (+/-0.17) | 1.24 (+/-0.28) | 1.54 (+/-0.18) | 1.00 (+/-0.15) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.518 | 0.402 | 0.207 | 1.34 (+/-0.23) | 1.00 (+/-0.17) | 1.04 (+/-0.18) | 1.72 (+/-0.64) | 0.94 (+/-0.27) | 1.04 (+/-0.24) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.545 | 0.708 | 0.154 | 1.25 (+/-0.13) | 0.83 (+/-0.19) | 1.15 (+/-0.1) | 1.18 (+/-0.13) | 1.05 (+/-0.21) | 1.22 (+/-0.16) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.333 | 0.934 | 0.223 | 1.10 (+/-0.14) | 0.89 (+/-0.09) | 1.00 (+/-0.12) | 1.12 (+/-0.08) | 0.81 (+/-0.14) | 1.52 (+/-0.37) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.587 | 0.552 | 0.823 | 0.99 (+/-0.19) | 1.01 (+/-0.16) | 1.16 (+/-0.09) | 1.27 (+/-0.18) | 1.02 (+/-0.16) | 2.11 (+/-0.65) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.856 | 0.498 | 0.196 | 1.15 (+/-0.12) | 0.82 (+/-0.06) | 1.08 (+/-0.04) | 1.19 (+/-0.19) | 1.04 (+/-0.15) | 1.33 (+/-0.25) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.467 | 0.614 | 0.755 | 1.50 (+/-0.33) | 1.53 (+/-0.64) | 1.28 (+/-0.24) | 1.40 (+/-0.21) | 1.45 (+/-0.2) | 2.11 (+/-0.69) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.163 | 0.552 | **0.006** | 1.37 (+/-0.12) | 0.65 (+/-0.08) | 1.13 (+/-0.12) | 1.18 (+/-0.17) | 0.95 (+/-0.11) | 1.14 (+/-0.17) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.507 | 0.714 | 0.167 | 1.08 (+/-0.13) | 0.85 (+/-0.2) | 0.77 (+/-0.08) | 0.97 (+/-0.08) | 0.83 (+/-0.08) | 1.08 (+/-0.18) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.644 | 0.597 | ***0.074*** | 1.30 (+/-0.18) | 0.76 (+/-0.07) | 1.22 (+/-0.17) | 1.22 (+/-0.21) | 0.97 (+/-0.2) | 1.30 (+/-0.17) |
| *Igf1* | insulin-like growth factor 1 | 0.8 | 0.677 | 0.193 | 1.17 (+/-0.2) | 0.94 (+/-0.2) | 0.98 (+/-0.2) | 1.57 (+/-0.26) | 1.29 (+/-0.46) | 1.09 (+/-0.24) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.842 | 0.389 | 0.466 | 1.02 (+/-0.1) | 0.76 (+/-0.14) | 1.01 (+/-0.23) | 1.24 (+/-0.41) | 0.78 (+/-0.11) | 0.92 (+/-0.11) |
| *Kiss1* | KiSS-1 metastasis-suppressor | 0.471 | 0.226 | 0.844 | 0.98 (+/-0.15) | 0.99 (+/-0.06) | 0.73 (+/-0.15) | 0.82 (+/-0.29) | 0.65 (+/-0.2) | 0.85 (+/-0.3) |
| *Kiss1r* | KISS1 receptor | 0.634 | 0.705 | 0.752 | 1.22 (+/-0.19) | 1.10 (+/-0.19) | 0.99 (+/-0.22) | 1.11 (+/-0.23) | 1.12 (+/-0.36) | 1.46 (+/-0.36) |
| *Lepr* | leptin receptor | 0.997 | 0.306 | 0.413 | 1.25 (+/-0.2) | 0.90 (+/-0.28) | 1.14 (+/-0.28) | 1.46 (+/-0.28) | 0.89 (+/-0.11) | 0.92 (+/-0.2) |
| *Mc3r* | melanocortin 3 receptor | 0.622 | 0.643 | 0.448 | 0.94 (+/-0.16) | 0.75 (+/-0.17) | 0.70 (+/-0.18) | 1.26 (+/-0.43) | 0.61 (+/-0.14) | 0.70 (+/-0.17) |
| *Nr3c1* | nuclear receptor subfamily 3, group C, member 1 | 0.484 | 0.764 | **0.014** | 1.20 (+/-0.11) | 0.70 (+/-0.09) | 0.92 (+/-0.08) | 1.11 (+/-0.13) | 0.88 (+/-0.07) | 0.99 (+/-0.15) |
| *Nrf1* | nuclear respiratory factor 1 | 0.993 | 0.794 | 0.541 | 1.21 (+/-0.15) | 0.90 (+/-0.06) | 1.10 (+/-0.12) | 1.41 (+/-0.36) | 1.19 (+/-0.27) | 1.49 (+/-0.38) |
| *Oxt* | oxytocin, prepropeptide | 0.865 | 0.635 | 0.656 | 1.11 (+/-0.29) | 1.19 (+/-0.47) | 0.56 (+/-0.12) | 0.65 (+/-0.14) | 7.49 (+/-6.82) | 0.67 (+/-0.13) |
| *Oxtr* | oxytocin receptor | 0.842 | 0.302 | 0.337 | 1.43 (+/-0.31) | 0.67 (+/-0.18) | 1.01 (+/-0.43) | 1.53 (+/-0.8) | 0.59 (+/-0.16) | 0.62 (+/-0.14) |
| *Per2* | period homolog 2 | 0.489 | 0.218 | 0.253 | 1.07 (+/-0.14) | 0.76 (+/-0.13) | 0.97 (+/-0.14) | 1.16 (+/-0.21) | 0.86 (+/-0.09) | 0.71 (+/-0.12) |
| *Pgr* | progesterone receptor | 0.572 | 0.622 | 0.342 | 1.24 (+/-0.16) | 0.79 (+/-0.2) | 0.77 (+/-0.16) | 0.88 (+/-0.26) | 0.84 (+/-0.13) | 0.90 (+/-0.24) |
| *Pomc* | proopiomelanocortin | 0.218 | 0.688 | 0.247 | 1.20 (+/-0.29) | 0.93 (+/-0.32) | 0.56 (+/-0.15) | 0.60 (+/-0.14) | 3.19 (+/-1.46) | 0.57 (+/-0.15) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 1 | ***0.091*** | 0.664 | 0.3 | 0.80 (+/-0.08) | 1.43 (+/-0.13) | 0.92 (+/-0.14) | 1.01 (+/-0.09) | 1.20 (+/-0.31) | 1.29 (+/-0.27) |
| *Tac2* | tachykinin 2 | 0.666 | 0.517 | 0.259 | 1.19 (+/-0.16) | 0.76 (+/-0.22) | 0.73 (+/-0.16) | 0.78 (+/-0.19) | 0.77 (+/-0.19) | 0.95 (+/-0.2) |
| *Tgfa* | transforming growth factor alpha | ***0.081*** | 0.593 | 0.274 | 1.20 (+/-0.1) | 0.98 (+/-0.08) | 0.98 (+/-0.08) | 1.01 (+/-0.2) | 1.30 (+/-0.15) | 0.94 (+/-0.13) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.375 | 0.529 | 0.743 | 1.04 (+/-0.13) | 1.08 (+/-0.12) | 1.53 (+/-0.33) | 1.19 (+/-0.21) | 1.29 (+/-0.32) | 0.99 (+/-0.18) |
| *Th* | tyrosine hydroxylase | 0.318 | 0.238 | 0.449 | 1.10 (+/-0.19) | 0.97 (+/-0.33) | 0.57 (+/-0.14) | 0.83 (+/-0.22) | 0.51 (+/-0.18) | 0.92 (+/-0.23) |

Table 3.3: Gene Expression in the Female Median Eminence (ME)

Relative gene expression for each treatment group is shown. P-values are listed for each variable. Significant main effects of treatment and status, or interaction of treatment x status are bolded ($p < 0.05$). Italics indicated that there was a trend ($p < 0.1$). Non-detectable genes are excluded from the table (*Crh,Cyp19a1, Dnmt3b, Dnmt3l, Drd4, Mc4r, Mc5r, Slc6a4*

| Gene Symbol | Gene Name | p-value Treatment | DMSO | EB | A1221 |
|---|---|---|---|---|---|
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.581 | 1.03 (+/-0.09) | 0.97 (+/-0.08) | 0.92 (+/-0.06) |
| *Ahr* | aryl hydrocarbon receptor | 0.825 | 1.57 (+/-0.26) | 1.57 (+/-0.24) | 1.37 (+/-0.26) |
| *Ar* | androgen receptor | 0.532 | 1.11 (+/-0.14) | 1.35 (+/-0.13) | 1.16 (+/-0.16) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.13 | 1.09 (+/-0.09) | 1.45 (+/-0.14) | 1.16 (+/-0.14) |
| *Avp* | arginine vasopressin | 0.265 | 2.43 (+/-0.98) | 16.53 (+/-8.27) | 3.38 (+/-1.85) |
| *Avpr1a* | arginine vasopressin receptor 1A | **0.05** | 0.75 (+/-0.1) | 1.24 (+/-0.19) | 0.93 (+/-0.11) |
| *Bdnf* | brain-derived neurotrophic factor | 0.713 | 0.83 (+/-0.13) | 0.90 (+/-0.11) | 0.76 (+/-0.12) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.408 | 1.32 (+/-0.27) | 1.86 (+/-0.36) | 1.46 (+/-0.23) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.428 | 1.13 (+/-0.1) | 1.30 (+/-0.13) | 1.09 (+/-0.1) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.474 | 1.35 (+/-0.15) | 1.60 (+/-0.21) | 1.31 (+/-0.15) |
| *Esr1* | estrogen receptor 1 | 0.739 | 0.92 (+/-0.17) | 1.11 (+/-0.18) | 0.98 (+/-0.16) |
| *Esr2* | estrogen receptor 2 | 0.331 | 0.81 (+/-0.11) | 0.98 (+/-0.12) | 0.78 (+/-0.07) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.401 | 1.55 (+/-0.22) | 1.49 (+/-0.14) | 1.23 (+/-0.14) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.259 | 1.02 (+/-0.1) | 1.25 (+/-0.09) | 1.01 (+/-0.11) |
| *Gal* | galanin prepropeptide | 0.115 | 0.88 (+/-0.19) | 1.52 (+/-0.26) | 1.08 (+/-0.18) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.541 | 1.34 (+/-0.14) | 1.25 (+/-0.13) | 1.13 (+/-0.14) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.961 | 1.04 (+/-0.16) | 1.08 (+/-0.22) | 0.99 (+/-0.26) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.112 | 1.03 (+/-0.08) | 1.28 (+/-0.12) | 1.03 (+/-0.08) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.473 | 1.06 (+/-0.11) | 1.28 (+/-0.14) | 1.13 (+/-0.11) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.547 | 1.13 (+/-0.1) | 1.34 (+/-0.17) | 1.19 (+/-0.13) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.928 | 1.41 (+/-0.12) | 1.44 (+/-0.18) | 1.36 (+/-0.16) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.67 | 1.21 (+/-0.11) | 1.30 (+/-0.1) | 1.16 (+/-0.1) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.868 | 1.35 (+/-0.12) | 1.35 (+/-0.13) | 1.26 (+/-0.18) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.584 | 1.13 (+/-0.09) | 1.23 (+/-0.15) | 1.04 (+/-0.13) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.237 | 1.06 (+/-0.1) | 1.41 (+/-0.14) | 1.25 (+/-0.15) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.823 | 1.15 (+/-0.11) | 1.12 (+/-0.05) | 1.06 (+/-0.13) |
| *Igf1* | insulin-like growth factor 1 | 0.825 | 0.71 (+/-0.09) | 0.75 (+/-0.07) | 0.66 (+/-0.11) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.267 | 1.18 (+/-0.1) | 1.31 (+/-0.17) | 1.03 (+/-0.1) |
| *Kiss1* | KiSS-1 metastasis-suppressor | 0.487 | 0.96 (+/-0.14) | 0.96 (+/-0.2) | 0.75 (+/-0.12) |
| *Kiss1r* | KISS1 receptor | 0.791 | 1.28 (+/-0.12) | 1.40 (+/-0.15) | 1.24 (+/-0.21) |
| *Lepr* | leptin receptor | 0.938 | 1.47 (+/-0.17) | 1.45 (+/-0.22) | 1.39 (+/-0.19) |
| *Mc3r* | melanocortin 3 receptor | 0.382 | 0.84 (+/-0.17) | 1.18 (+/-0.18) | 0.89 (+/-0.15) |
| *Mc4r* | melanocortin 4 receptor | 0.571 | 1.09 (+/-0.16) | 1.13 (+/-0.19) | 0.90 (+/-0.14) |
| *Oxt* | oxytocin, prepropeptide | 0.171 | 1.32 (+/-0.58) | 3.74 (+/-1.81) | 1.32 (+/-0.32) |
| *Oxtr* | oxytocin receptor | 0.264 | 0.89 (+/-0.11) | 1.18 (+/-0.13) | 0.95 (+/-0.13) |
| *Pdyn* | prodynorphin | 0.477 | 0.75 (+/-0.11) | 0.96 (+/-0.17) | 0.77 (+/-0.1) |
| *Per2* | period homolog 2 | 0.115 | 0.89 (+/-0.1) | 1.38 (+/-0.23) | 1.00 (+/-0.15) |
| *Pgr* | progesterone receptor | 0.792 | 0.93 (+/-0.16) | 1.08 (+/-0.17) | 0.95 (+/-0.15) |
| *Slc17a6* | solute carrier family 17, member 6 | 0.714 | 1.05 (+/-0.15) | 1.18 (+/-0.2) | 0.99 (+/-0.13) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.943 | 1.59 (+/-0.21) | 1.56 (+/-0.23) | 1.49 (+/-0.21) |
| *Tac2* | tachykinin 2 | 0.197 | 0.99 (+/-0.17) | 1.50 (+/-0.23) | 1.16 (+/-0.17) |
| *Tgfa* | transforming growth factor, alpha | 0.584 | 1.08 (+/-0.1) | 1.26 (+/-0.13) | 1.09 (+/-0.15) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.919 | 1.26 (+/-0.1) | 1.20 (+/-0.13) | 1.28 (+/-0.14) |
| *Thra* | thyroid hormone receptor, alpha | 0.599 | 1.20 (+/-0.1) | 1.31 (+/-0.13) | 1.13 (+/-0.12) |
| *Thrb* | thyroid hormone receptor, beta | 0.463 | 1.26 (+/-0.11) | 1.27 (+/-0.13) | 1.09 (+/-0.12) |

Table 3.4: Gene Expression in the Male Anteroventral Periventricular Nucleus (AVPV)

Relative gene expression for each treatment group is shown. P-values are listed for each variable. Significant main effects are bolded ($p < 0.05$). Italics indicated that there was a trend ($p < 0.1$). Non-detectable genes are excluded from the table (*Dnmt3l, Gnrhr, Mc5r*).

| Gene Symbol | Gene Name | p-value Treatment | DMSO | EB | A1221 |
|---|---|---|---|---|---|
| *18S* | Eukaryotic 18S RNA, 18S ribosomal 1 | 0.43 | 0.99 (+/-0.05) | 1.00 (+/-0.09) | 0.87 (+/-0.09) |
| *Ahr* | aryl hydrocarbon receptor | **0.006** | 1.18 (+/-0.14) | 1.49 (+/-0.18) | 0.84 (+/-0.06) |
| *Ar* | androgen receptor | 0.462 | 0.95 (+/-0.08) | 1.10 (+/-0.06) | 0.97 (+/-0.1) |
| *Arntl* | aryl hydrocarbon receptor nuclear translocator-like | 0.409 | 1.01 (+/-0.12) | 1.06 (+/-0.1) | 0.85 (+/-0.11) |
| *Avp* | arginine vasopressin | 0.265 | 1.58 (+/-0.27) | 1.85 (+/-0.37) | 0.80 (+/-0.12) |
| *Avpr1a* | arginine vasopressin receptor 1A | 0.963 | 1.03 (+/-0.13) | 0.99 (+/-0.11) | 0.99 (+/-0.12) |
| *Bdnf* | brain-derived neurotrophic factor | **0.008** | 0.90 (+/-0.13) | 2.14 (+/-0.35) | 1.42 (+/-0.26) |
| *Cyp19a1* | cytochrome P450, family 19, subfamily A, polypeptide 1 | 0.232 | 1.00 (+/-0.18) | 2.75 (+/-1.07) | 1.07 (+/-0.14) |
| *Dnmt1* | DNA (cytosine-5-)-methyltransferase 1 | 0.464 | 1.14 (+/-0.15) | 1.08 (+/-0.11) | 0.90 (+/-0.15) |
| *Dnmt3a* | DNA (cytosine-5-)-methyltransferase 3a | 0.667 | 1.19 (+/-0.17) | 1.19 (+/-0.19) | 1.02 (+/-0.1) |
| *Esr1* | estrogen receptor 1 | 0.642 | 0.92 (+/-0.08) | 0.84 (+/-0.09) | 0.96 (+/-0.09) |
| *Esr2* | estrogen receptor 2 | 0.587 | 1.10 (+/-0.12) | 0.96 (+/-0.07) | 0.96 (+/-0.11) |
| *Gad1* | glutamate decarboxylase 1 (brain, 67kDa) | 0.758 | 1.18 (+/-0.16) | 0.97 (+/-0.04) | 1.02 (+/-0.08) |
| *Gad2* | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | 0.669 | 0.96 (+/-0.11) | 0.86 (+/-0.08) | 0.86 (+/-0.07) |
| *Gal* | galanin prepropeptide | 0.6 | 0.69 (+/-0.11) | 0.77 (+/-0.12) | 0.61 (+/-0.08) |
| *Gapdh* | glyceraldehyde-3-phosphate dehydrogenase | 0.469 | 0.96 (+/-0.14) | 1.02 (+/-0.09) | 0.83 (+/-0.09) |
| *Gnrh1* | gonadotropin-releasing hormone 1 | 0.515 | 0.73 (+/-0.15) | 0.60 (+/-0.14) | 0.85 (+/-0.14) |
| *Gnrhr* | gonadotropin-releasing hormone receptor | 0.83 | 0.80 (+/-0.17) | 0.67 (+/-0.14) | 1.41 (+/-0.78) |
| *Gper* | G protein-coupled estrogen receptor 1 | 0.411 | 1.33 (+/-0.16) | 1.47 (+/-0.1) | 1.18 (+/-0.15) |
| *Gria1* | glutamate receptor, ionotropic, AMPA 1 | 0.421 | 0.98 (+/-0.12) | 1.06 (+/-0.06) | 0.93 (+/-0.08) |
| *Gria2* | glutamate receptor, ionotropic, AMPA 2 | 0.198 | 0.93 (+/-0.12) | 1.22 (+/-0.11) | 0.95 (+/-0.1) |
| *Grik2* | glutamate receptor, ionotropic, kainate 2 | 0.5 | 1.27 (+/-0.18) | 1.30 (+/-0.14) | 1.08 (+/-0.1) |
| *Grin1* | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 0.822 | 1.07 (+/-0.11) | 1.10 (+/-0.06) | 1.01 (+/-0.08) |
| *Grin2a* | glutamate receptor, ionotropic, N-methyl D-aspartate 2A | 0.381 | 1.22 (+/-0.13) | 1.47 (+/-0.11) | 1.24 (+/-0.13) |
| *Grin2b* | glutamate receptor, ionotropic, N-methyl D-aspartate 2B | 0.943 | 1.10 (+/-0.16) | 1.12 (+/-0.08) | 1.06 (+/-0.11) |
| *Grin2c* | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 0.374 | 0.91 (+/-0.14) | 1.07 (+/-0.07) | 0.86 (+/-0.07) |
| *Grin2d* | glutamate receptor, ionotropic, N-methyl D-aspartate 2D | 0.824 | 1.00 (+/-0.11) | 1.00 (+/-0.08) | 0.93 (+/-0.08) |
| *Igf1* | insulin-like growth factor 1 | 0.298 | 0.96 (+/-0.16) | 1.09 (+/-0.19) | 0.74 (+/-0.12) |
| *Igf1r* | insulin-like growth factor 1 receptor | 0.183 | 1.08 (+/-0.11) | 1.13 (+/-0.1) | 0.89 (+/-0.07) |
| *Kiss1* | KiSS-1 metastasis-suppressor | 0.827 | 0.45 (+/-0.07) | 0.42 (+/-0.05) | 0.40 (+/-0.05) |
| *Kiss1r* | KISS1 receptor | 0.537 | 1.08 (+/-0.11) | 1.30 (+/-0.16) | 1.12 (+/-0.14) |
| *Lepr* | leptin receptor | 0.936 | 1.13 (+/-0.17) | 1.16 (+/-0.15) | 1.09 (+/-0.1) |
| *Mc3r* | melanocortin 3 receptor | 0.751 | 0.96 (+/-0.14) | 1.05 (+/-0.16) | 0.90 (+/-0.11) |
| *Mc4r* | melanocortin 4 receptor | 0.335 | 1.35 (+/-0.33) | 0.81 (+/-0.1) | 1.14 (+/-0.19) |
| *Oxt* | oxytocin, prepropeptide | ***0.091*** | 0.76 (+/-0.14) | 1.24 (+/-0.25) | 0.52 (+/-0.07) |
| *Oxtr* | oxytocin receptor | 0.82 | 0.67 (+/-0.16) | 0.82 (+/-0.18) | 0.70 (+/-0.15) |
| *Pdyn* | prodynorphin | 0.446 | 0.77 (+/-0.14) | 1.00 (+/-0.16) | 0.77 (+/-0.11) |
| *Per2* | period homolog 2 | 0.159 | 0.98 (+/-0.11) | 1.03 (+/-0.12) | 0.78 (+/-0.06) |
| *Pgr* | progesterone receptor | 0.82 | 0.58 (+/-0.05) | 0.58 (+/-0.05) | 0.54 (+/-0.06) |
| *Slc17a6* | solute carrier family 17, member 6 | ***0.057*** | 0.87 (+/-0.12) | 1.32 (+/-0.07) | 1.01 (+/-0.14) |
| *Srd5a1* | steroid-5-alpha-reductase, alpha polypeptide 2 | 0.682 | 1.19 (+/-0.15) | 1.27 (+/-0.12) | 1.11 (+/-0.1) |
| *Tac2* | tachykinin 2 | 0.718 | 0.49 (+/-0.09) | 0.43 (+/-0.05) | 0.52 (+/-0.07) |
| *Tgfa* | transforming growth factor, alpha | 0.346 | 0.89 (+/-0.08) | 1.05 (+/-0.09) | 0.92 (+/-0.07) |
| *Tgfb1* | transforming growth factor, beta 1 | 0.105 | 0.98 (+/-0.05) | 1.25 (+/-0.14) | 1.01 (+/-0.08) |
| *Thra* | thyroid hormone receptor, alpha | 0.714 | 1.06 (+/-0.13) | 1.12 (+/-0.07) | 0.99 (+/-0.09) |
| *Thrb* | thyroid hormone receptor, beta | 0.417 | 1.05 (+/-0.11) | 1.11 (+/-0.09) | 0.93 (+/-0.07) |

Table 3.5: Gene Expression in the Male Arcuate Nucleus (ARC)

Relative gene expression for each treatment group is shown. P-values are listed for each variable. Significant main effects are bolded ($p < 0.05$). Italics indicated that there was a trend ($p < 0.1$). Non-detectable genes are excluded from the table (*Dnmt3l, Mc5r*).

| | | DMSO | EB | A1221 |
|---|---|---|---|---|
| | p-value | Mean SEM | Mean SEM | Mean SEM |
| **Body Wt (g)** | 0.969 | 562.5 (+/- 21.58) | 556.44 (+/- 11.33) | 558.9 (+/- 12.53) |
| **Pituitary Wt (g)** | 0.364 | 0.014 (+/- 0.) | 0.013 (+/- 0.001) | 0.0132 (+/- 0.) |
| **Adrenal Wt (g)** | 0.987 | 0.05 (+/- 0.002) | 0.049 (+/- 0.002) | 0.049 (+/- 0.002) |
| **GSI** | 0.961 | 0.007 (+/- 0.) | 0.008 (+/- 0.) | 0.008 (+/- 0.) |
| **Serum LH (ng/ml)** | 0.478 | 0.455 (+/- 0.054) | 0.633 (+/- 0.196) | 0.444 (+/- 0.029) |
| **Serum T (ng/ml)** | 0.364 | 0.474 (+/- 0.058) | 0.459 (+/- 0.139) | 0.649 (+/- 0.125) |
| **Serum E2 (pg/ml)** | **0.022** | 2.36 (+/- 0.264) | ***7.72 (+/- 2.968)*** | ***4.13 (+/- 0.589)*** |
| **AVPV Avpr1a** | **0.05** | 0.751 (+/- 0.097) | **1.25 (+/- 0.191)** | 0.932 (+/- 0.109) |
| **AVPV Ahr** | **0.006** | 1.18 (+/- 0.14) | **1.49 (+/- 0.182)** | 0.842 (+/- 0.058) |
| **AVPV Bdnf** | **0.008** | 0.895 (+/- 0.129) | **2.14 (+/- 0.353)** | 1.42 (+/- 0.257) |
| **ARC Slc17a6** | ***0.057*** | 0.872 (+/- 0.119) | 1.32 (+/- 0.073) | 1.01 (+/- 0.139) |
| **ARC Oxt** | ***0.091*** | 0.756 (+/- 0.137) | 1.24 (+/- 0.247) | 0.519 (+/- 0.069) |

Table 3.6: Effects of prenatal EDCs on somatic markers and hormones in male rats.

Those genes with significant differences or trends in AVPV or ARC are shown. No significant effects of prenatal EDC treatment on somatic markers were detected. For serum hormones, only $E_2$ was affected, with higher concentrations in both EB and A1221 males. For gene expression, three significant effects were detected, one in AVPV and 2 in ARC, with differences between EB males and their DMSO counterparts. We also present data for two genes for which there were trends in the ARC.

## DISCUSSION

Endocrine disrupting chemicals have become a prototypical model for the "fetal basis of adult disease," with prenatal exposures during critical developmental periods associated with adult dysfunctions related to reproductive, metabolic, thyroid, and many other target systems. However, little of this research has extended the "fetal basis" model to aging populations, and an even smaller subset on reproductive senescence. The current study sought to determine if gestational exposure to low doses of estrogenic EDCs would hasten reproductive aging in males and females through premature aging of neuroendocrine function, assayed here through a network of gene expression in three key hypothalamic regions. The basis for this work was the body of epidemiological evidence suggesting that there are correlations between exposures to EDCs and advancements in

the timing of menopause in humans (Akkina et al., 2004; Cooper et al., 2002; Hatch et al., 2006; Knox et al., 2011). However, some of these results have been contradictory (Akkina et al., 2004; Cooper et al., 2002; Farr et al., 2006; Hatch et al., 2006; Knox et al., 2011; Yu et al., 2000), possibly due to differences in exposure timing, dose, and type of EDC. Animal models may be more useful in determining the cause-and-effect links between early life EDCs and reproductive aging. Our laboratory recently found that perinatal exposure to the pesticide methoxychlor advanced reproductive senescence in Fischer female rats and altered gene expression and DNA methylation in the hypothalamus 16 months after the original exposure (Gore et al., 2011). Other rodent work shows that EDCs can cause irregular estrous cyclicity in females and accelerate the transition to persistent estrus by 9 months of age (Adewale et al., 2009; Armenti et al., 2008; Dickerson et al., 2011b; Dickerson and Gore, 2007; Gellert, 1978a, b). Previous studies specifically investigating the effects of A1221 on the timing of reproductive aging in female rats (Gellert, 1978a, b; Gellert and Wilson, 1979) have produced inconsistent results and did not investigate molecular endpoints. While Gellert et al (Gellert and Wilson, 1979) investigated gestational exposure to A1221 on male fertility at 6 months of age, to our knowledge no study has investigated the long-term hypothalamic effects of A1221 on male rodents.

Based on the literature to date, our experimental design sought to address several novel points. First, we tested how ecologically-relevant exposures to estrogenic EDCs might affect reproductive physiology in males and females. There is a paucity of data on sex differences caused by EDCs beyond the early parts of life. Second, we characterized

physiological changes in the animals throughout their first 9 months of life, representing a significant portion of a rat's lifecycle, and associated them with molecular endpoints in brain regions important for reproductive function and physiology. Third, we performed cluster and network analyses to gain insights into the complex relationships caused by prenatal EDC exposure – this goes beyond the "gene by gene" or "hormone by hormone" approach and has been very informative about the big picture of bionetworks (Ojeda et al., 2010a; Walker et al., 2012).

**Reproductive physiology is altered in females exposed to A1221 in utero**

While we did not see a significant advancement of the timing of reproductive senescence or in the number of acyclic females by 9 months of age, we did observe a significant increase in the length of estrous cycles in A1221 females (Figure 3.2) throughout the life cycle. Elongated cycles are a common characteristic of reproductive aging in both primates and rodents before transitioning to acyclicity (Kermath and Gore, 2012) and the appearance of such cycles earlier in life may be indicative of an aging phenotype. Additionally, we noted a marked decreased (albeit non-significant) in serum LH in cycling females exposed perinatally to A1221 (Figure 3.1A) when compared to their control counterparts. The pulsatile nature of LH release probably led to high variability in serum LH concentrations between individuals. In rodents, age-related decreases in LH release on proestrus presage reproductive decline, and are associated with decreased GnRH drive (Lloyd et al., 1994; Zuo et al., 1996). Further studies are necessary to test whether this is the case in prenatally EDC-exposed female rats.

**Gestational exposure to A1221 alters cycle status specific expression of neuroendocrine genes in the ARC and ME, but not the AVPV, of female rats**

The ARC plays a role in the regulation of GnRH pulsatility (Reviewed in (Navarro, 2012)), and in negative feedback of hormones on GnRH/LH release, processes that are subject to age-related decline (Weiss et al., 2004). This region had the greatest number of genes that were affected by EDC treatment interacting with cycle status. Interestingly, gene expression tended to be higher in acyclic than cycling rats in the control DMSO groups, whereas opposite effects were seen for the A1221 rats (gene expression was lower in acyclic than cyclic). EB rats tended to fall in the middle (Figure 3.3). These genes fell into 4 functional categories: epigenetic processes, steroid hormone receptors, neuropeptide signaling, and glutamate signaling. While changes in hypothalamic sex steroid hormone feedback and glutamatergic signaling are thought to contribute to the transition to reproductive senescence (Reviewed in (Gore, 2001)), it should be noted that many of these genes are important for signaling cross-talk among neuroendocrine systems regulating reproduction, stress, and metabolism (e.g., *Pdyn, Lepr, Mc3r* and *Oxt*) (Onaka et al., 2012; Shimizu et al., 2007; Yosten and Samson, 2010a, b). All of these axes undergo age related changes in rodents (Bowman et al., 2006; Santollo et al., 2012) and non-human primates (Reviewed in (Appt and Ethun, 2010)), and our data suggest that gestational exposure to A1221 causes lifelong alterations that may affect numerous neuroendocrine axes. Furthermore, our data provide insight into target gene candidates that may play roles in the transition from cyclicity to acyclicity.

In the ME, we found that levels of steroid hormone receptor genes for ERα and the glucocorticoid receptor (*Esr1, Nr3c1*) and neurotransmitter systems (*Grin2b, Gad2*)

were affected by prenatal EDCs (Figure 3.4). In DMSO rats, acyclic females had lower expression of all four of these genes compared to cycling DMSO rats (notably, this is the opposite gene pattern from the ARC). Again, A1221 rats had completely different gene expression patterns by cycle status from the DMSO group. The ME is mainly composed of glial cells and axons projecting to the portal capillary system to release neuropeptides (Yin and Gore, 2010); the low occurrence of neuronal cell bodies in this region suggests that the gene expression results in this experiment are probably mainly from glial cells or local mRNA in axons (Reviewed in (Jung et al., 2012)). The few studies on reproductive aging investigating changes specifically in the ME revealed age-related changes: Direct stimulation of GnRH release from terminals with glutamate or glutamate agonists was decreased in middle aged females compared to young (Zuo et al., 1996), and the ultrastructural organization of the ME, and the relationship between glia and GnRH terminals in this region, was disrupted with age (Yin et al., 2009a; Yin et al., 2009b).

It was surprising that so few effects of aging or cycle status were found in AVPV. It is possible that nine months may be too early in reproductive aging to see an effect. In comparing results for the three hypothalamic regions, there are distinct regional differences in responses to prenatal EDCs, and in effects of cycle status. Overall, results from the ARC and ME indicate that patterns of gene expression between cyclic and acyclic rats seen in control treatments are obliterated or even reversed in A1221 rats. Although this requires further experimentation, we hypothesize that A1221 females begin the transition of reproductive aging earlier in life and that the overall process is altered. This hypothesis was supported by our hierarchical cluster analysis, whereby acyclic

A1221 females cluster with the cycling DMSO females and vice versa in both the ARC and ME. The elongated cycles and lower serum levels of LH in A1221 cycling rats further support this idea. Finally, expression patterns of genes in the ARC and ME in cycling females exposed to A1221 are similar to the acyclic, and dissimilar to the cyclic, control rats.

Our data must be interpreted in light of some limitations. All rats were euthanized at a single time of day, and it is possible that the treatment effects observed on gene expression are due to other influences such as a disruption of circadian rhythms or changes to other endocrine organs that feedback onto the hypothalamus. Although we were able to assay multiple genes, serum hormones, and somatic endpoints, several additional endpoints (e.g. expression and localization of proteins in the brain; release of neurotransmitters/ neuropeptides) would be extremely valuable information. Finally, the age of euthanasia (9 months) is fairly early in the aging process for the females and males, and later ages would be very informative. Nevertheless, even at 9 months, it is clear that there are distinct differences between the prenatal treatment groups in our aging population.

**Housing conditions may alter effects of treatment in males and females**

In general, there were very few effects of treatment in males on somatic indices, serum hormones, and gene expression in the ARC and AVPV. Previous studies from our laboratory are consistent with this latter result, and showed that exposure to A1221 during the critical period alters programming of the female hypothalamus to a much greater extent than in males (Dickerson et al., 2011a; Dickerson et al., 2011b). However,

we have observed that exposure to A1221 on E16 and 18 delays the timing of puberty in males (Dickerson et al., 2011b). A sister study from our laboratory found that gene expression is altered specifically in the male ARC throughout adulthood (unpublished data). Again, the lack of effects of A1221 here may be due to the relatively early age at euthanasia (9 months).

We believe that the housing conditions of our rats may have had unexpected and potentially interesting influences on some outcomes, that merit further investigation. This experiment was part of a larger study in which littermates had been euthanized at earlier life ages for a companion experiment. At P90, rats for the current aging study were randomly paired with a new same-sex cage mate. Possible housing pairs were DMSO-DMSO, DMSO-EB, EB-EB, DMSO-A1221, A1221-A1221, and EB-A1221, but because investigators were blind to treatment, some combinations were overrepresented and others were underrepresented (in fact, we never had any DMSO-DMSO pairings for either sex). While these data should be considered preliminary, an individual's partner had effects on body weight, gene expression, and cycle status. For example, males housed with an EB treated cage mate weighed less than the EB cage mate, whereas those housed with a DMSO cage mate showed no differences in their weights (data not shown). Interestingly, genes associated with social behaviors were affected in AVPV and ARC of males and may relate to the housing conditions. There is some evidence that social environment can affect the timing of reproductive aging in male mice (Schmidt et al., 2009). While this clearly needs further investigation it provides intriguing evidence that

effects of treatment may be exacerbated or masked by an interaction with social condition.

In females, we noted that 69% of the cage mates had the same cycling status as their partners suggesting that cycling status may be sensitive to social condition in the aging females, as reported previously (Hermes et al., 2009; Hermes and McClintock, 2008; Hermes et al., 2006). This is a plausible explanation for the high number of acyclic females treated with DMSO, as they were all housed with EB or A1221 partners. We had expected that the treated females (both EB and A1221) would have a greater number of acyclic females than our control group (Gellert, 1978b; Gore et al., 2011) and the similarities in acyclic and cyclic animals of all treatments may relate to the social pairing condition.

**Network analysis reveals region-specific changes between cycling and acyclic females**

In order to generate new hypotheses about hypothalamic and peripheral changes during the transition of reproductive aging, we used Cytoscape (Kohl, 2011; Kohl et al., 2011; O'Connell and Hofmann, 2012) to generate networks of hormones, genes and endocrine tissues in each of the regions of interest. The most striking difference between cyclic and acyclic networks was seen in the AVPV. This result was both interesting and surprising, as the gene-by-gene analysis did not reveal gene expression effects, whereas the network analysis gave a more holistic perspective on patterns of change with aging. In the cycling animals there were 3 "hubs" of negative correlations: serum estradiol ($E_2$), adrenal weights, and uterine weights. However, in the acyclic females the number of

significant correlations was substantially decreased, and virtually all negative relationships were with serum LH concentrations. Because the AVPV is necessary for the LH surge (Wiegand and Terasawa, 1982), it is not surprising that we saw stark differences between the cyclic and acyclic network. Moreover, it should be noted that the correlations observed in the cycling AVPV reveal some unexpected relationships. Notably, serum $E_2$ and *Pgr* (progesterone receptor) were negatively correlated in contrast to other studies (Blaustein et al., 1988; Blaustein and Turcotte, 1989; Shughrue et al., 1997). Most work on regulation of the progesterone receptor by $E_2$ is conducted on models of ovariectomy +/- estradiol treatment. Our current study on intact females provides new information about changes in this relationship with aging. We recently published (Walker et al., 2012) a paper showing that during early postnatal development of intact female rats, the $E_2$-*Pgr* connection also differs from the OVX model, suggesting that models of natural development and aging with a bionetwork approach is important in understanding physiological regulatory processes.

In the ARC, one of the more surprising findings was that the cycling animals had a hub of positive correlations with Gnrh1 and a negative hub with gonadal somatic index (ovary) weights. GnRH cell bodies of rats are primarily located in the AVPV but not the ARC of the rodent (King et al., 1982). However, these data provide intriguing evidence of local translation of *Gnrh1* in the axons of the GnRH neurons and may be reflective of the ready, releasable pool of GnRH. While this has not been observed in GnRH neurons specifically, other neuroendocrine cells, e.g. oxytocin and vasopressin, use local translation to maintain and replenish neuropeptide levels at the site of release (Jung et al.,

2012). In the acyclic rat's ARC, the hubs of positive correlations were *Gnrhr*, pituitary and adrenal weights whereas the negative hub is uterus weight. It is notable that many of the genes that were altered by treatment in the ARC are associated with the 2 positive hubs in the cyclic and acyclic networks suggesting these genes may have differing roles in the cyclic and acyclic ARC. Interestingly, two of these somatic endpoints (pituitary and uterus) are the same ones that show significant effects of status independent of treatment supporting the ARC's role in the central regulation of reproductive aging.

In the ME, there were few differences in the number of correlations between cyclic and acyclic end points. Additionally, there do not appear to be distinct hubs as observed in the AVPV and the ARC. Many of the correlations were between genes and not peripheral changes, which was surprising since the ME is the region of final output of the GnRH system to the HPG axis. The reduced number of correlations found and lack of hubs in the ME networks compared to the AVPV and ARC may signify a different role in reproductive function. With the presence of numerous neurons in the AVPV and ARC, many of which modulate GnRH neuronal activation and release, the coordination of their activity may be aptly reflected in a network of gene expression. However, while changes in gene expression are still likely important at the site of the terminals in the ME, the coordination of regulatory inputs (or "network") may be better demonstrated at the protein level, as many neuropeptides and transmitters are released there.

## SUMMARY AND CONCLUSIONS

Numerous studies have indicated that gestational exposure to EDCs can disrupt reproductive function and results in premature reproductive aging. Our study sought to

characterize the long-term changes reproductive physiology and gene expression throughout the hypothalamus in an aging model in both males and females. First, we found very few effects in the males either expression or the somatic markers of aging. In the females, however, animals exposed to A1221 underwent a different process of reproductive aging than their control counterparts. Additionally, we identified genes in both the ARC and ME as potential targets of EDCs as well as biomarkers for altered reproductive aging. Finally, we used bionetworks to identify relationships of genes, hormones, and somatic changes that can provide insight into the process of reproductive aging and serve as hypothesis generating tools for future experiments on reproductive senescence.

# GENERAL CONCLUSIONS

Taken together, the experiments presented here provide a comprehensive developmental profile of neuroendocrine gene expression changes in the hypothalamus and serum hormone concentrations from the neonate through reproductive aging in males and females. Although each experiment did not look at every age, I was able to replicate previous work throughout all experiments. However, my studies were far more comprehensive in that they included animals of both sexes at a range of ages; littermates were distributed across different time points to avoid over-reliance of siblings at a single age; numerous genes could be measured within individuals; the hypothalamus was subdivided to provide better anatomical specificity; and serum hormones were measured in the same animals. This design allowed me to explore new possibilities regarding the mechanisms of sexual differentiation of the brain as well as functional differences in the regulation of reproductive physiology between brain regions.

In addition, my dissertation compared normative hypothalamic development to that of animals exposed to prenatal EDCs. This is important because few studies have investigated changes in genes and hormones in the same animals throughout development and there are vast differences in experimental design between these studies. This made it difficult to interpret any effects of EDCs as we did not have a consistent basis for comparison

In chapter 1, I investigated the relationships between hormones and gene expression in the hypothalamus of normally developing intact male and female rats throughout postnatal development and into adulthood. This experiment revealed novel

relationships between genes and hormones in two regions of the hypothalamus. I also reported that there were surprisingly few sex differences in the POA and MBH, with only *Esr1*, *Kiss1* and *Tac2* being sexually dimorphic. The sex differences were specific to brain regions and observed at specific ages. This result was important because all three of these genes are necessary for reproductive function and are expression in the same neurons in the MBH. Until recently, it was thought that these genes were not sexually dimorphic in those neurons. However, our data and others reveal novel sex difference in the caudal hypothalamus.

In chapter 2, I investigated if gestational exposure to PCBs, a known class of endocrine disrupting chemicals, altered the development of the hypothalamus. Here, I further refined my hypothalamic dissection and used microdissected anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of males and females from the juvenile period (P15) through the pubertal process (P30 and P45) and into adulthood (P90). I noted significant treatment effects in the females of 21 genes in the AVPV, including the same three genes highlighted in Chapter 1. Additionally, the expression profiles of 9 genes in the female AVPV were masculinized in their expression. This result suggests that gestational exposure to estrogenic EDCs can alter the developmental process in a male typical pattern. These effects were associated with altered estrous cyclicity in adult females, indicating a functional consequence of the prenatal EDC exposures. In males, the timing of puberty was delayed by A1221 treatment. I also observed alterations of adrenal development, slight effects on gonadal somatic index, and serum LH concentrations at specific ages. While there were few

effects in the male AVPV, 6 genes were altered by treatment in the ARC, most notably *Ar* and *Lepr*. Taken together, these data suggest that ARC plays an important role in the timing of puberty in males, whereas the AVPV plays an important role in regulating the estrous cycle in females.

I believe it is important that two of the genes altered by treatment in the female AVPV, *Per2* and *Arntl*, function as clock genes and regulate circadian expression of other genes. Recent publications suggest that Kiss1 is under circadian control in the female AVPV. My data are the first to show that clock gene expression is sexually dimorphic in the AVPV and can be programmed by gestational exposure to estrogenic EDCs. These data also provide the first evidence that sexually dimorphic expression of genes in the AVPV may be under circadian control and are dependent on the cycle status of the female, thereby highlighting the importance of including intact, cycling animals when investigating the neuroendocrine control of reproductive physiology.

In chapter 3, I extended my analysis of gestational exposure to EDCs to the other end of the life spectrum, by analyzing effects on reproductive senescence in aging females and males. Siblings of the animals from the experiment in chapter 3 were allowed to age until 9 months of age. While there were few treatment effects observed in males, the females exhibited disrupted estrous cycles, altered LH concentrations, and effects on expression of genes in the ARC and median eminence (ME). However, unlike the young animals, no effects of cycle status or treatment were observed in the AVPV. Additionally, there were no differences in the number of acyclic females in the treated groups when compared to the DMSO group. In order to gain further understanding of

changes in the brain during the transition to reproductive senescence, I generated bionetworks to represent changing relationships between genes, hormones and endocrine tissues in cycling and acyclic. From these bionetworks I was able to develop specific hypotheses about region-specific changes during reproductive aging. Overall, my data suggested that changes in expression of genes in the AVPV preceded the transition to reproductive senescence, and resulted in altered estrous cycles, even before females became acyclic. However, it is seemingly the ARC and ME that undergo changes resulting in acyclicity and infertility as there were a number of genes altered in the ARC and ME when comparing cyclic and acyclic expression profiles.

In the next sections, I will discuss why I think these results are biologically relevant, and discuss specific data that emphasize the points mentioned above.

**IMPLICATIONS OF THIS WORK**

Taken together, my data provide evidence for three overarching themes in regard to reproductive neuroendocrinology: 1) intact animals do not display relationships in gene expression and hormones that have been traditionally reported. In fact, my results of developmental patterns of serum sex steroid hormones ($E_2$, T, $P_4$) bear little relationship to gene expression when compared to published studies investigating hormone regulation of gene expression in gonadectomized models. 2) Network analysis revealed that the AVPV relates to sexually dimorphic temporal regulation of GnRH/LH release, and the ARC to the preovulatory GnRH/LH surge and the timing of reproductive transitions. More specifically, females in my studies exposed to EDCs in utero were more likely to have disrupted estrous cycles and a number of genes altered in the AVPV. Notably,

numerous genes exhibited a male-typical expression profiles from P15 – P90. However, the ARC was relatively unaffected. On the other hand, in both chapters 2 and 3 reproductive transitions were affected by gestational exposure to EDCs. In males, the timing of puberty was delayed and in females the process of reproductive aging was altered. In both cases, the effects of EDCs on gene expression were observed in the ARC. 3) I identified novel targets of endocrine disruption as well as several genes that are consistent targets of gestational exposure to EDCs. With respect to identifying new targets, to my knowledge, I am the first to report that *Per2* and *Arntl* are sexually dimorphic in the AVPV and can be programmed via gestational exposure to estrogenic compounds. Additionally, *Ar* was consistently altered by gestational exposure to EDCs, in agreement with other studies from my laboratory (Dickerson et al., 2011b; Gore, 2008). This suggests that *Ar* is a potent target of A1221, presumably through its estrogenic actions as similar effects were caused by EB, my estrogenic control.

**Intact animals do not display relationships in gene expression and hormones that have been traditionally reported**

In general, few of the predicted relationships between genes and serum hormone concentrations were observed in young animals or aging females. For example, as *Pgr* is well-established as being regulated by $E_2$ (Lauber et al., 1991b, c; Quadros et al., 2002; Quadros and Wagner, 2008), I was expecting that this would be observed throughout my developmental studies. However, this was never the case in my intact rats. In the young animals, at no age was a significant positive correlation observed between serum $E_2$ with the estrogen receptors or with *Pgr* (Chapter 1 Figure 1.5 and 1.6). Notably, in the cyclic

AVPV of the 9mo old females, $E_2$ was negatively correlated with *Pgr* as well as *Ar* and *Esr1* (Chapter 3, Figure 3.6). These discrepancies were also observed for *Kiss1* expression, which is positively regulated by serum $E_2$ in the AVPV and negatively regulated by serum $E_2$ in the ARC (Reviewed in (Oakley et al., 2009)). However, in my developing animals *Kiss1* expression was positively correlated with $P_4$ in the POA after puberty (P45 and P60) and was often negatively correlated with serum T. In the MBH, *Kiss1* was not significantly correlated with any serum hormone concentrations. Interestingly, the aging females, *Kiss1* in the cyclic AVPV was positively correlated with *Avp* (a gene involved in circadian signaling from the SCN) and in the cyclic ARC was positively correlated with *Gnrh*. The one time I observed a predicted correlation with *Kiss1* was in the ARC of acyclic females, in which *Kiss1* and *Tac2* were negatively correlated with serum $E_2$. As a whole, these studies reveal novel and complex relationships between serum hormone concentrations and gene expression that are specific to age, sex and cycle status and brain region. They also highlight the necessity for further studies to include intact and cycling animals in experiments to enable a fuller understanding of reproductive neuroendocrinology.

**Effects of prenatal EDCs in the AVPV**

One of the most surprising findings of these studies was that Kiss1 expression was not sexually dimorphic in the AVPV in adult animals. Additionally, in my treated animals, *Kiss1* expression was greater in the female AVPV when compared to the males on P90 (Chapter 2, Figure 2.1). I noted similar expression patterns of genes previously reported to be sexually dimorphic in the AVPV but only when females were in diestrus or

estrus but not proestrus (*Esr1* (Zhou et al., 1995) and *Pdyn* (Simerly et al., 1996)). My females were euthanized during their predicted preovulatory GnRH/LH surge on proestrus (1 – 3 hours before lights out). These alterations in gene expression, along with notable changes in estrous cyclicity (especially the observation that treated animals had elongated periods of estrus and diestrus), suggest that the timing of the surge may be disrupted in the animals exposed to EB and A1221. My observation that the two clock genes on my array (*Per2* and *Arntl*) were affected by treatment in a sexually dimorphic manner, with a masculinized pattern in females, is concordant with this as the results suggest a temporal misalignment of hypothalamic gene expression and reproductive physiology. Nevertheless, our females were still had LH surges and were fertile (data not shown). These data suggest that disruptions in the AVPV, even of genes known to be necessary for reproductive function can be overcome.

Results from my aging study showed that the estrous cycles were elongated and more likely to be irregular, similar to what was observed for young adult females with prenatal EDC exposure. Therefore, I hypothesize that sexually dimorphic gene expression is reduced in the AVPV of females as they progress into reproductive senescence. I propose a model by which circadian genes in the AVPV such as *Per2* and *Arntl* lose their sexually dimorphic expression due to the prenatal endocrine disruption (Conclusions, Figure 4.1). My finding of a marked reduction in sexually dimorphic expression of *Per2* and *Arntl* in the AVPV as females progressed from cyclic to acyclic supports this prediction. There is evidence that circadian responsiveness declines with aging in male rodents (Biello, 2009). In women, reproductive aging is associated with a marked

reduction in morning $E_2$ and $P_4$ surges (Ahn et al., 2011) and the LH surge is delayed and attenuated in the aging female rodent (Scarbrough and Wise, 1990). Taken together, these data indicate that there is circadian component to reproductive aging that is masculinized by early life EDC exposures.

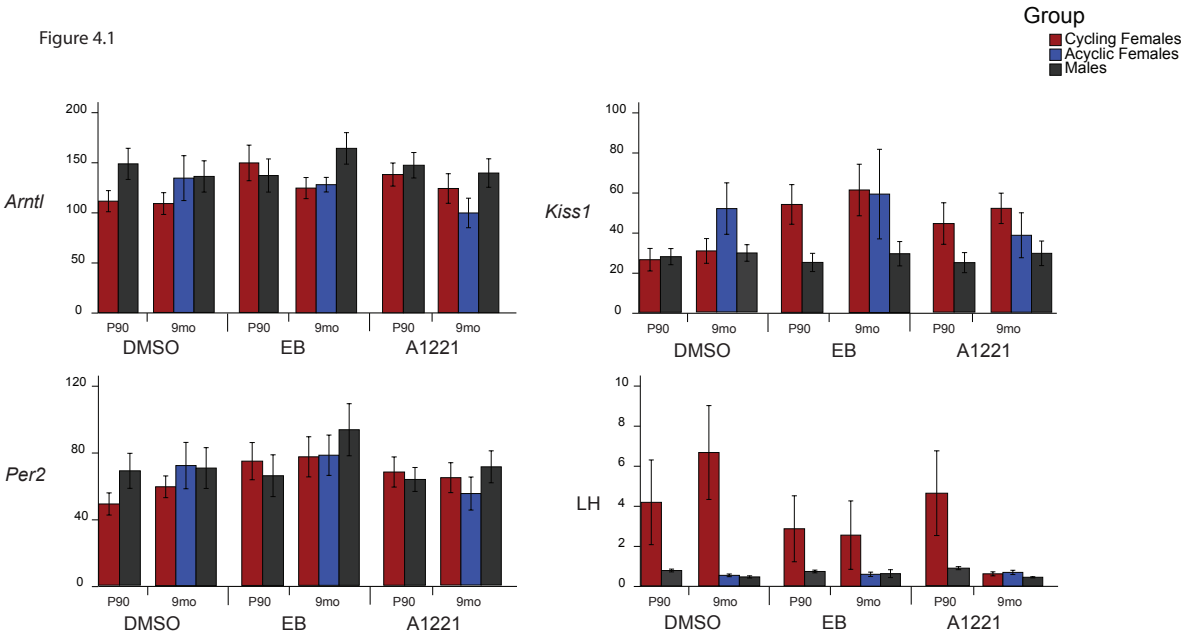



Figure 4.1: Comparison of sexually dimorphic expression in the adult & aging AVPV.

Cycling females are indicated in red, acyclic females are in blue (9mo only) and males are in black. Because both our treated and aged females had elongated cycles, we compared expression of Arntl, Per2 and Kiss1 to males in both ages to see if sexually dimorphic expression was decreased between cyclic and acyclic females. For all 3 genes, our DMSO acyclic females displayed similar expression to our young treated females and males. Serum LH is included as an indicator of the preovulatory surge.

**Effects of prenatal EDCs in the ARC**

Another surprising finding replicated throughout my experiments was that *Kiss1* and *Tac2* were sexually dimorphic in the ARC. Recent evidence suggests that the KNDy neurons in the ARC of ewes are important for generating the preovulatory LH surge.

Furthermore, Ruiz et al, reported that *Tac2* is sexually dimorphic in the KNDy neurons in mice (Ruiz-Pino et al., 2012). The fact that the KNDy neurons were not disrupted by EDCs and our females were still having LH surges and estrus cycles suggests that the ARC may also play a role in the pre-ovulatory surge. I propose that synchrony between the AVPV and ARC is important for the precise regulation of the release of LH, which in turn results in regular estrous cycles and a precisely timed LH surge. However, dysregulation of the system does not lead to infertility but rather unpredictable timing of the LH surge. This is a plausible explanation for the differences previously observed in reproductive behaviors in females exposed to A1221. Our laboratory and others have reported that exposure to A1221 results in females being less receptive (Chung and Clemens, 1999) and requiring more trials before successfully mating (Steinberg et al., 2008). If the LH surge was shifted or attenuated due to a decoupling of circadian signals from the AVPV and "pacemaking" signals from the ARC, the females may be "out of phase" with the males attempting to mate with them.

My results support the possibility that the timing of reproductive transitions involves the ARC. This is supported by my findings that males exposed to EDCs *in utero* went through puberty later than their control counterparts, and that the most robust effects on gene expression in males were observed in the ARC. However, it is difficult to know the causality, i.e., if peripheral changes preceded changes in gene expression, or vice versa. What can be said is that *Ar* and *Lepr* are not simply responding to changes in serum T concentrations as changes in serum T and gene expression are not consistent

throughout development or treatment. However, there are age specific gene expression changes between treatment groups.

In the aging study, I noted that a majority of the effects of treatment on gene expression were in the ARC and ME of females, either cycling or acyclic. Again, it is difficult to determine if gene expression changes are initiating the timing of reproductive senescence or if they are occurring in response to peripheral changes. However, there were no differences in serum $E_2$ between cyclic and acyclic females, suggesting that the gene expression differences were not in response to fluctuations in $E_2$. Other hormones and peripheral factors may be involved, something that requires further study. While the exact role of the ARC in reproductive transitions is not well understood, my data provide intriguing evidence that the ARC is involved in this process. Future studies should address this question from the perspective of the AVPV and the ARC, together, as these two brain regions are likely to interact in the control of reproductive development and senescence.

**Novel targets of endocrine disruption, notably aspects of the circadian clock, and molecules that are consistent targets of gestational exposure to EDCs**

I identified several novel genes that were affected by gestational exposure to EDCs. Most notably were the 2 clock genes included on our array, *Per2* and *Arntl*. Additionally, I found that *Ar* was consistently affected by treatment in several brain regions including the AVPV of my young females, the ARC of our young and aged males, and the ARC of the aging females. Interestingly, studies have reported sex differences in the circadian system and there is strong evidence for the role of *Ar* in

regulating circadian behaviors. For example, AR protein expression in mice is greater in the male SCN when compared to the female. Additionally, gonadectomy reduces activity in the evening (ZT 12) in males but has no effect on female behavior, which can be restored with testosterone replacement (Iwahana et al., 2008). In gonadectomized but not intact or DHT treated male mice, light-induced mPER2 protein expression is decreased in the evening (ZT13.5), and light-induced mPER1 expression is increased late at night (ZT 21) suggesting that the AR is involved in regulating clock genes in the SCN. It has been proposed that sex differences in *Ar* expression in the SCN are necessary to ensure activity coincides with the time of ovulation in females (Karatsoreos et al., 2011). This is supported by the finding that there are sex differences in PER2 protein expression in the SCN (to my knowledge other clock genes have not been investigated in the brain), although it should be noted that those differences are dependent on the estrus cycle. For example, peak *Per2* gene expression in SCN explants from females is at ZT13.5 on proestrus and metestrus (Nakamura et al., 2010). PER2 protein expression in the SCN shows a similar peak at ZT 13. However, there are sex differences in the rate of decline after lights out. On proestrus and estrus, PER2 protein expression decreases rapidly and reaches a nadir by 6 hours after lights out. On metestrus and diestrus expression is similar to males with levels peaking at the time of lights out and remaining elevated for 6 hours. Interestingly, gonadectomy in both sexes results in an expression pattern that is similar to females in proestrus and estrus (Perrin et al., 2006).

While previous studies have focused on the SCN, it should be noted that I saw similar sex differences in *Per2* expression in the AVPV of the DMSO animals

(euthanized between ZT 9 – 11). In my animals, *Arntl*, *Per2* and *Ar* expression in the treated females were similar to males throughout development. A similar finding was made for *Gnrh1*, which displays sex differences in circadian expression in the POA (Gore, 1998; Gore and Roberts, 1995). However, numerous other genes were not masculinized by treatment but instead displayed a female-typical expression pattern if the female was in diestrus rather than proestrus. For example, *Kiss1* expression was sexually dimorphic in AVPV of treated animals (Chapter 2 Figure 2.1). Data from my aging study suggests that arginine vasopressin (*Avp*) is an important regulator of cyclicity in the AVPV as it is the only "hub" that is a gene in our bionetworks of the cycling females. Interestingly, Avp is one of the 2 main signaling molecules from the SCN thought to regulate circadian GnRH release, the other being vasoactive intestinal protein (Vip). Unfortunately, my PCR low-density array did not include Vip or its receptor. However, I did find that vasopressin receptor expression (Avpr1a) was altered by treatment in the female AVPV. Furthermore, expression was almost identical to that of Kiss1, with sex differences only observed in the treated animals.

Thus, I hypothesize that gestational exposure to estrogenic endocrine disrupting chemicals results in the circadian clock being less sensitive to resetting by central and peripheral cues. This could lead to a phase shift in the LH surge and a decoupling of precise timing of activity, the preovulatory GnRH/LH surge, and reproductive behavior in females. Furthermore, this shift is similar to what is observed in middle-aged females undergoing the process of reproductive senescence. Further studies are necessary to provide more conclusive evidence. It is necessary to investigate numerous time points

throughout the day, and to perform studies in the absence of diurnal cues (e.g. in constant dark). Physiological and behavioral measures of circadian rhythmicity would need to be measured. Additionally, follow-up analysis should be conducted in the SCN, together with the AVPV and ARC, to add more comprehensive information about SCN and other central nervous system clocks. Analyses should include genes involved in circadian rhythms such as *Vip* and its receptor and a broader array of clock transcription factors.

## CONCLUDING REMARKS

Taken together, my dissertation provides novel insight into the mechanisms of basic neuroendocrinology, reproductive transitions and endocrine disruption. By approaching the subject of endocrine disruption from a developmental perspective, we were able to assess novel targets of EDCs and identify specific ages when exposure might be having the most profound effects. These may be useful as biomarkers of gestational EDCs exposure or may also be helpful in identify developmental time points when the effects of EDCs could be mediated. Finally, by comparing a disrupted phenotype to untreated and DMSO control profiles, I was able to develop new hypothesis regarding the functional roles of well characterized nuclei of the hypothalamus. Finally, in the human population, symptoms of gestational exposure to EDCs (Schug et al., 2011) closely resemble those identified in sleep deprived including increased risk of metabolic disorder (Penev, 2007), cancer (Davis et al., 2001) and infertility (Nurminen, 1998). Although further study is necessary, these data provide intriguing evidence for a novel mechanism of endocrine disruption.

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